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TITLE: Biomarkers in the Detection of Prostate Cancer in African Americans

PRINCIPAL INVESTIGATOR: William E. Grizzle, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 35294

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14. ABSTRACT

All IRBs have been approved by the participating sites and the DOD. Dr. Grizzle's laboratory has completed a pilot analysis by Luminex multiplex immunoassay of serum using two panels of biomarkers, cancer biomarkers (e.g., CA19.9) and inflammatory cytokines (e.g., IL-17a), to identify potential biomarkers that are differentially expressed. Molecular features that are not differentially expressed and for which there are not apparent racial differences will be excluded in future studies to narrow the biomarkers to be evaluated on the larger series of cases. In collaboration with Dr. Clayton Yates at Tuskegee, microRNAs have been identified that apparently vary with race. A study of prostate cancer is now underway to identify microRNAs that are differentially expressed in cancer and that show racial differences. Dr. Gaston's laboratory has completed a pilot analysis of DNA methylation markers that are sensitive to the presence of occult high grade prostate cancer that's been missed due to biopsy sampling error. Our collaborator Rick Kittles and others have shown that cancer-associated DNA methylation marker patterns differ in patients of different racial ancestry. We are now well positioned to utilize our DNA methylation marker "field effect" test in a comparison of our AA and EA study subjects.

15. SUBJECT TERMS
Prostate cancer, molecular markers, multiplex immunoassays, racial differences

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INTRODUCTION

African Americans (AAs) are reported to have a poorer outcome from prostate cancer (PCa) than do European Americans (EAs), yet there have been few studies which have evaluated/compared the differences in molecular features of PCas of AA patients with the molecular features of PCas that develop in EA patients. Our goal is to identify molecular differences between PCa from AAs and from EAs. We hypothesize that molecular features that differ between PCas from AAs and from EAs may be markers of aggressiveness or rapid progression. Such biomarkers could be used in determining prognosis and/or as targets for small molecules or immunotherapy to reduce the aggressiveness of PCas from any race. Our approach, to discover biomarkers of PCa that are differentially expressed between PCas from AAs versus from EAs based on racial admixture data to complement self declared race, will utilize Affymetrix and/or liquid chromatography mass spectrometry (LCMS) and will be confirmed independently by TLDA RT-Q-PCR. Initially, mRNA and protein will be extracted from nitrocellulose blots of biopsies of the prostate. In a separate analysis, PCa from AAs will be compared with PCa from stage and Gleason score matched PCa from EAs that are age matched (± 5 years). We also will determine the racial admixtures of 150 AAs and 150 EAs from the Alabama population evaluated for prostatic diseases using archival tissues including buffy coats and TaqMan Low Density Arrays with RT-Q-PCR will be used to confirm the racial differential expression candidate genes of PCa identified by the initial studies. A separate multiplex analysis of serum will be used to identify molecular markers of PCa differentially expressed based on race and/or presence of cancer. This study brings together laboratories at two research institutions, UAB (Grizzle, P.I.) and Tufts University/New England Baptist Hospital (Gaston, P.I.) in a project which would not be completed by either laboratory alone.

BODY

To date, we have completed the performance by Luminex multiplex immunoassay of two panels of molecular markers using serum from 18 patients with prostate cancer and 19 patients without prostate cancer. One panel of analytes includes 37 cytokines and other inflammatory molecules, while the other panel includes 23 analytes that are commonly used tumor markers such as CA19.9. There is some overlap (5 analytes) that were analyzed in the two panels. The first goal of this pilot analysis is identifying the molecular markers that look promising (Table 1) in the analysis of serum from patients with prostate cancer (PCa). Those molecules that appear not to be promising (Table 2) may be excluded in the design of new panels of molecular markers for the analysis of the molecular features of serum from patients with prostate cancer unless racial differences are identified. Analysis of racial differences will soon be complete. In Figure 1, the performance of free PSA is demonstrated. As would be expected, free PSA is increased in patients who do not have prostate cancer. A ratio of free PSA/PSA could not be evaluated because the panel does not permit total PSA and free PSA to be performed in the same assay. Therefore, we selected free PSA for this first assay. In Figure 2, the performance of epidermal growth factor (EGF) is evaluated demonstrating increased expression in serum from patients with PCa. In Figure 3, the performance of interleukin 6 is demonstrated. It does not show differential expression but does indicate a potential racial difference. In our analysis, potential racial differences of molecular features that do not seem to be differentially expressed in comparing patients with PCa and those without PCa (Table 2) will be studied further. For example, GRO (Figure 4) indicates a likely racial difference between AA and EA patients with cancer. To confirm any indications of racial differences, a new set of samples will be evaluated.

The pilot immunoassay results from PCa were surprising in that they differ extensively from our experience; previously, we had observed in immunoassays of pancreatic cancer that molecular markers were in general higher in the tumor compared to non-cancer (e.g., pancreatitis (ref 1). In contrast, based on the pilot study (Table 1), most of the potential molecular markers of interest in PCa are higher in non-cancer. In general, this may result in a more difficult identification of the molecular markers in cancer cases because it is in general not as easy to detect down regulation of a molecular feature at the tissue level, an approach we planned to use to verify the expression of serum markers in tissues. These results may indicate that benign prostatic hyperplasia (BPH) which most of the non-cancer cases may have resulting in an increased PSA in non-cancer cases, may increase selected molecular markers secondary to increased volume of the prostate in BPH. Thus, this may require more emphasis on the more specific cancer associated molecules such as EGF and soluble FAS ligand which are elevated in the cases of cancer.

We have developed a collaboration with Dr. Clayton Yates at Tuskegee University to evaluate racial differences in selected microRNAs in prostate cancer. Dr. Yates previously had identified that there are racial differences in the expression of some microRNAs in breast and prostate cancer. We identified 20 cases of prostate cancer in AA and 20 cases of prostate cancer in CA patients. We extracted microRNAs from paired matching areas of cancer and areas of

uninvolved prostatic tissue on each case (80 specimens). The microRNAs of interest have been analyzed using RT-Q-PCR. The results appear encouraging and statistical analysis of the data is underway. Results will be reported in the next quarterly report.

Related Data to Support the Purpose of This Grant: Many studies have shown that tumor-associated changes in DNA methylation can act as biomarkers for the presence of prostate cancer. We and others have also shown that changes in DNA methylation patterns often occur in histologically benign tissues adjacent to cancer, thus acting as "field effect" biomarkers. As part of a study supported by the NCI Early Detection Research Network (EDRN), we have used tissue prints to evaluate diagnostic prostate biopsies to determine if the field effects generated by cancer-associated DNA methylation might serve as a useful test for the presence of occult prostate cancer in the tissues adjacent to the biopsy core. We have identified three DNA hypermethylation markers (GSTPi, APC and RASSF1A) with field effects that extend far enough around the histological boundary of a prostate cancer focus to be detected in a biopsy core several millimeters away. Importantly, for two of these markers (GSTPi and APC), we found that both the magnitude and the extent of field-effect hypermethylation are sensitive to the grade of the adjacent prostate cancer. These data were presented at the American Urological Association Annual Meeting in May 2011 (copy attached). This last year, Dr. Gaston's laboratory has extended these studies with an analysis of prostate biopsies from more 70 subjects to evaluate a methylation marker "signature" that is sensitive to the presence of high grade prostate cancer in adjacent tissues (cancer that was missed due to biopsy sampling error). This prototype "field effect" test is particularly timely as more patients are asked to consider active surveillance rather than immediate treatment based on a biopsy diagnosis of low grade prostate cancer.

We have not yet compared DNA methylation field effect markers in African American and European patients, but published studies report that that for several DNA markers the extent of hypermethylation is higher in prostate cancers from AAs. Moreover, there is evidence that processes that produce racial differences in DNA methylation may arise early in life. It should be noted that Dr. Rick Kittles, who is a collaborator on this DOD project, is a co-author of a study published in May 2012 comparing tumor DNA methylation in AA and EA breast cancer patients (refs 2-4). This study suggests that differential DNA methylation patterns in AA and EA breast cancer may represent an integration of lifestyle and genetic predisposing factors resulting in altered patterns of gene expression and differences in clinical outcome and behavior. Inasmuch as our plan of work for DOD project PC093309 already calls for the preparation of DNA samples for the analysis of ancestry informative markers, we are now well positioned to utilize our DNA methylation marker protocols (a series of methylation specific qPCRs) in a comparison of our Birmingham AL AA and EA study subjects. Our general approach to this grant is discussed in an abstract (attached) presented at the 2011 AACR Conference on Cancer Health Disparities.

KEY RESEARCH ACCOMPLLISHMENTS

- Completed a pilot assay of serum from patients with and without prostate cancer using Luminex multiplex immunoassays of two panels of inflammatory cytokines/molecules and of tumor markers.
- Completed analysis of immunoassays of prostate cancer as to differential expression of molecules as to cancer versus non-cancer and have begun analysis as to racial differences.
- Identified 20 AA and 20 EA patients with prostate cancer; microRNAs were extracted from matched areas of cancer and non-cancer from each case (paraffin embedded tissues). These were provided to the laboratory of Dr. Clayton Yates, Tuskegee University, to confirm his observations as to racial differences in microRNAs. Results are encouraging and are under statistical analysis.
- Completed a pilot analysis of a set of DNA methylation markers that are sensitive to the presence of occult high grade prostate cancer that has been missed due to biopsy sampling error. We are now well positioned to utilize our DNA methylation marker "field effect" test in a comparison of our Birmingham AL AA and EA study subjects.

REPORTABLE OUTCOMES

- Administrative: Dr. Gaston's IRB has been approved (September 2012, copy attached) by Tufts University and by the DOD (copy attached); therefore, we will now complete the material transfer agreement (MTA) between UAB and Tufts University. The MTA could not be developed prior to IRB approvals. This is a major administrative advance in our efforts to begin our work in Dr. Gaston's laboratory. With IRB approvals, we should be able to complete the MTA rapidly (few weeks).

- A book chapter on exosomes in cancer has been published (A) and a review manuscript (Biotech Histochem) on post transcriptional processing and cancer is at the page proofs stage (B). Additionally, three review manuscripts have been published in Cancer Biomarkers (C-E). These are "Biomarkers and the genetics of early neoplastic lesions," "The biology of incipient, pre-invasive or intraepithelial neoplasia," and "Translational pathology of neoplasia." These manuscripts are included in the appendix.

CONCLUSION

One challenge is the question of whether there are any molecular changes that have occurred during storage in paraffin tissue sections that we previously cut from paraffin blocks for analysis of racial admixtures. These sections have been stored at 4ºC for over six months. When 4µ paraffin sections are cut and stored, there is a reduction in immunorecognition after more than several days of storage (ref 2). Thus, after approval of Dr. Gaston's IRB by the DOD, we will randomly select 30 AA and 30 CA patients and re-cut their cases so that the analysis of the stored tissue aliquots (paraffin sections) can be compared with the same analysis of newly cut paraffin sections. Although we expect there to be no differences, this becomes a very important control.

In the next quarter, an independent set of 37 serum samples enriched in African Americans with prostate cancer will be used to verify our initial observations on those molecular features that are promising as to differential expression in cancer or with respect to race. The results of this set should permit the design of our serum multiplex immunoassays for prostate cancer.

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2. Relationship between tumor DNA methylation status and patient characteristics in African-American and European-American women with breast cancer. Wang S, Dorsey TH, Terunuma A, Kittles RA, Ambs S, Kwabi-Addo B. PLoS One. 2012;7(5):e37928.
3. Kwabi-Addo B, Wang S, Chung W, Jelinek J, Patierno SR, Wang BD, Andrawis R, Lee NH, Apprey V, Issa JP, Ittmann M. Identification of differentially methylated genes in normal prostate tissues from African American and Caucasian men. Clin Cancer Res. 2010 Jul 15;16(14):3539-47
4. Adkins RM, Krushkal J, Tylavsky FA, Thomas F. Racial differences in gene-specific DNA methylation levels are present at birth. Birth Defects Res A Clin Mol Teratol. 2011;91(8):728-36.
5. Manne U, Myers RB, Srivastava S, **Grizzle WE**. Re: Loss of Tumor Marker-Immunostaining Intensity on Stored Paraffin Slides of Breast Cancer. J Natl Cancer Inst 1997; 89(8):585-586.

SUPPORTING DATA

Table 1: Molecules that Warrant Additional Study Based on Differential Levels of the Molecule in Serum (> 25% Difference)				
	Differential Expression		Higher In	
Molecule	Uninvolved Median Value	Cancer Median Value	Uninvolved	Cancer
EGF	176	326		X
Free PSA	815	479	X	
Exotaxin	195	131	X	
Fractalkine	94	50	X	
IFNa2	28	14	X	
IFNδ	3	0.2	X	
IL-1a	10	2	X	
IL-1b	1.9	0.1	X	
IL-1ra	35	11	X	
IL-3	0.8	0.03	X	
IL-2	3.2	1.1	X	
IL-5	2.5	0.5	X	
IL-6	4.9	2.6	X	
IL-7	7.0	4.2	X	
IL-8	15	9	X	
IL-9	1.8	0.9	X	
IL-10	9.6	3.8	X	
IL-12 (p40)	35	15	X	
IL-13	2.4	1.0	X	
IL-17A	1.4	0.5	X	
IP-10	467	350	X	
MCP-3	41	20	X	
MDC	1201	1714		X
SCD40L	671	168	X	
TNFβ	5.7	0.4	X	
He-4	1315	154	X	
OPN	178	120	X	
CEA	1383	1788		X
SFASL	1.0	4.6		X
Leptin	16.6	11.3	X	

Table 2: Molecules that May Not Warrant Additional Study in Serum Based on Lack of Differential Expression			
Molecule	**Uninvolved Median Value**	**Cancer Median Value**	**Comments**
FGF2	52	40	
TLT-3L	2	2	
G-CSF	51	36	
GM-CSF	21	13	Marginal
GR0	918	822	Potential Racial Difference
IL-4	30	36	
IL-6	4.0	3.7	Potential Racial Difference
IL-12 (p70)	1.5	1.6	
MCP-1	510	493	
MIP-1a	11	12	
MIP-1b	71	80	
TGFα	6.3	7.2	
TNFα	10	8.4	
b-HCG	0.2	0.22	
CYFRA21-1	50	50	
SCF	91	72	
Prolactin	6005	6616	
SFAS	3700	3651	
HGF	415	389	
TRAIL	113	107	
MIF	2110	2486	
CA19.9	17.3	20.3	
CA15.3	27	27	
AFP	3450	3450	
vEGF	137	167	

Figure 1

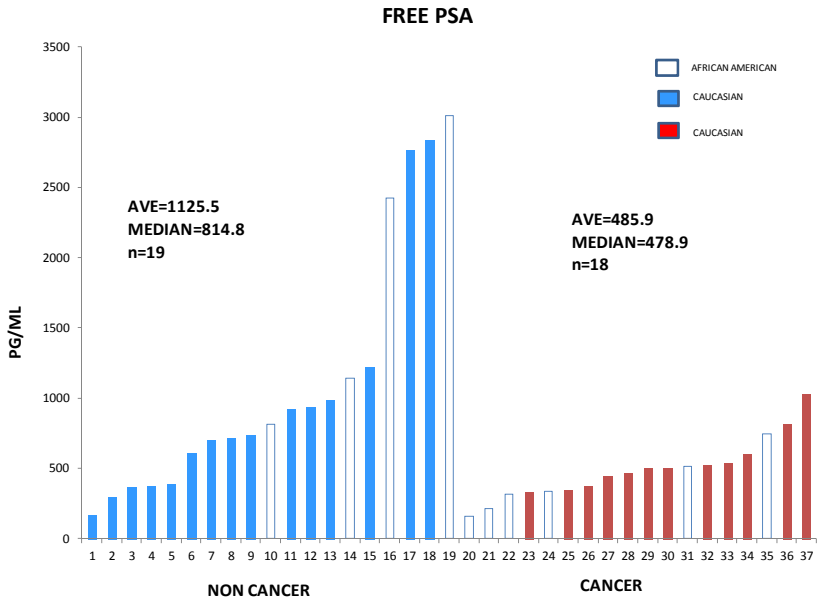

Figure 1: This graph demonstrates values in serum of free prostatic specific antigen (PSA) organized from low to high levels in each of two groups, patients with biopsy proven prostate cancer and patients whose biopsies did not demonstrate cancer. As would be expected, free PSA is much higher in patients who do not have a biopsy which indicates cancer. The distribution in cancer cases does not suggest a racial difference.

Figure 2

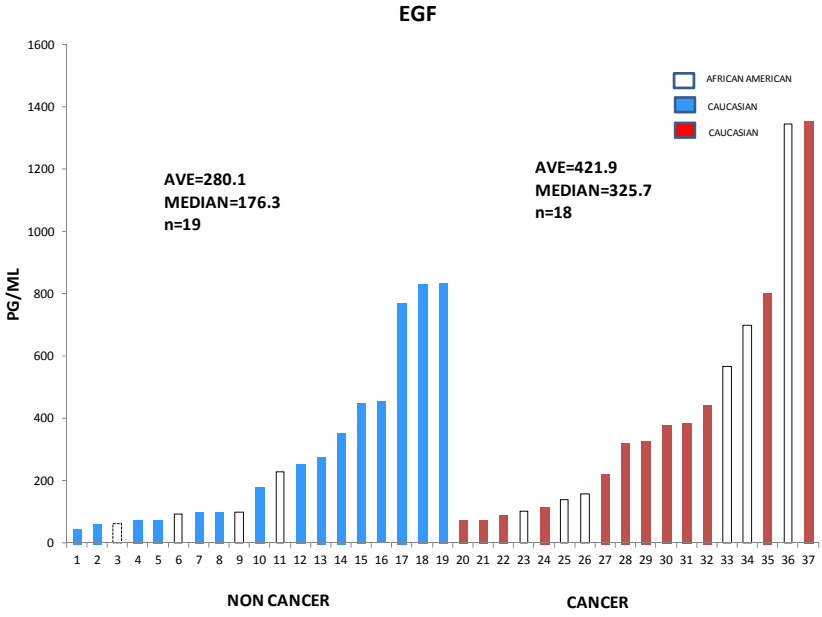

Figure 2: The organization of this graph is similar to Figure 1. Serum levels of epidermal growth factor (EGF) are much higher in patients with prostate cancer and there is a suggestion of higher levels having a tendency of being obtained from AAs.

Figure 3

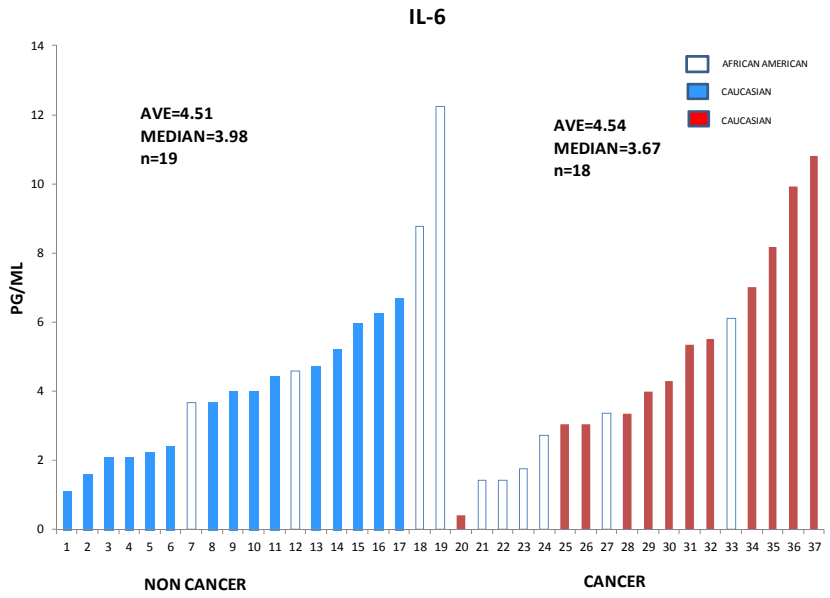

Figure 3: Serum levels of interleukin 6 are very similar in cancer and non-cancer patients. There appears to be a slight difference based on racer (higher levels in non-cancer and lower levels in cancer.

Figure 4

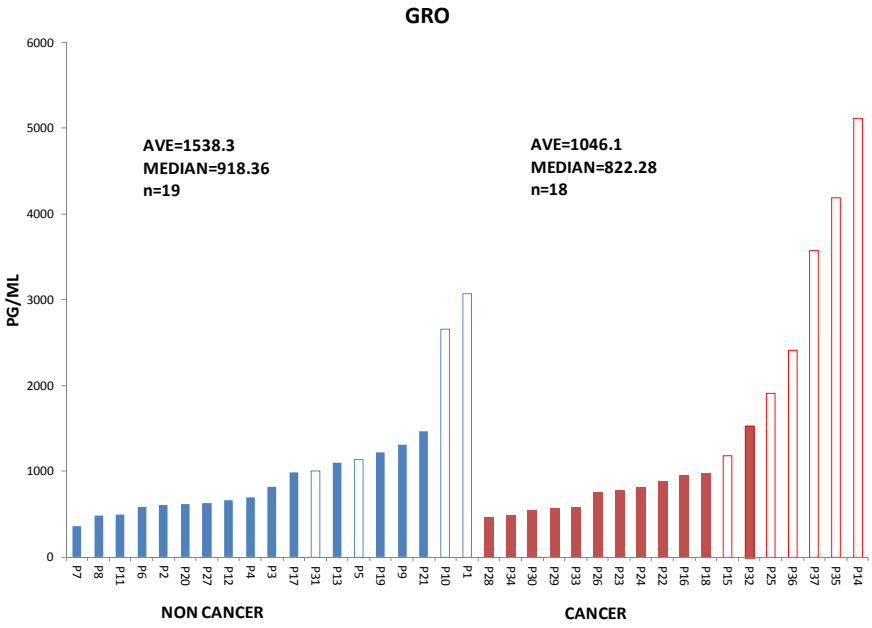

Figure 4: Serum levels of GRO (CXCL 1, 2 & 3) are very similar in cancer and non-cancer; however, there is a clear racial difference in the distribution of these molecular markers.

APPENDICES

Tufts IRB Approval (Dr. Sandra Gaston)

DOD Approval of Tufts IRB (Dr. Sandra Gaston)

Cummulative Publications Resulting from this DOD Grant (Copies included in Appendix):

A. Zhang HG, **Grizzle WE**. The effects of exosomes and related vesicles on cancer development, progression and dissemination. *In: Emerging Concepts of Tumor Exosome-Mediated Cell-Cell Communication,* (Eds HG Zhang), Springer Science. 2012;107-129.

B. McNally LR, Manne U, **Grizzle WE**. Post-transcriptional processing of genetic information and its relation to cancer. Biotech Histochem (in press).

C. Srivastava S., **Grizzle WE**. Biomarkers and the genetics of early neoplastic lesions. Cancer Biomark 2011;(9)(1-6):41-64. NIHMSID:NIHMS402044.

D. **Grizzle WE**, Srivastava S, Manne U. The biology of incipient, pre-invasive or intraepithelial neoplasia. Cancer Biomark 2011;9(1-6):21-39. NIHMSID:NIHMS402045.

E. **Grizzle WE**, Srivastava S, Manne U. Translational pathology of neoplasia. Cancer Biomark 2011;9(1-6):7-20. NIHMSID: NIHMS402047.

F. **Gaston SM,** Guerra AL, Grooteclaes M, Renard, I, Kearney MC, Bigley J and Kearney GP. Gene Methylation Biomarker Analysis of Prostate Biopsies from Men with 1 of 12 Cores Positive for Cancer: Greater Methylation Prevalence and Extent in Gleason 7 than Gleason 6 Cancer. American Urological Association Annual Meeting, Washington DC; May 2011.

G. **Gaston SM**, Kearney GP, **Grizzle WE**. Prostate biopsy tissue print technologies; a practical and innovative approach to overcoming racial disparities in the datasets used for prostate cancer biomarker development. Presented at the AACR Cancer Health Disparities Meeting, Washington, D.C., September 18, 2011.

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DOCUMENTATION OF EXEMPT STATUS

Sandra Gaston, PhD
Tufts MC, Box 802
Boston, MA 02111

IRB #: 10538
Protocol Title: Biomarkers in the detection of prostate cancer in African Americans

Date of IRB Determination: 09/12/12

In accordance with 45 CFR 46.101(b)(4) the Tufts Medical Center/Tufts University Health Sciences IRB determined that the above-referenced project is exempt.

The IRB made the following findings:

- Health Insurance Portability and Accountability Act documentation is not required; per the Principal Investigator, the data will be de-identified.

The exempt status of this research will not expire. Please notify the IRB office in writing when this project is terminated.

Any change to this project that may affect the exempt status of this project must be submitted to the IRB for review prior to implementation.

THIS NOTICE MUST BE RETAINED WITH YOUR FILES.

9/12/12
Date

KLEIN, MD
Signature of Chair/Vice Chair/Designee

/JJ

OMB No. 0990-0263
Approved for use through 1/31/2012

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule. Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[X] ORIGINAL [ ] CONTINUATION [ ] EXEMPTION	[X] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	
4. Title of Application or Activity **Biomarkers in the detection of prostate cancer in African Americans**		5. Name of Principal Investigator, Program Director, Fellow, or Other **Gaston, Sandra PhD** **Pathology** **Tufts MC** **IRB #: 10538**

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. **FWA00004449** the expiration date **5/24/2014** IRB Registration No. **IORG0000435**

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[ ] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting) _____ or [X] Expedited Review on (date) **9/12/2012**
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments

This project was deemed to be Exempt in accordance with 45 CFR 46.101(b)(4) at Tufts University/Tufts Medical Center.

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* **617.636.7512** 12. Fax No. *(with area code)* **617.636.8394** 13. Email: **AKlein2@tuftsmedicalcenter.org**	**Tufts University Health Sciences** **800 Washington Street** **Boston, MA 02111**
14. Name of Official **Andreas K. Klein, MD**	15. Title **IRB Chair**
16. Signature A Klein	17. Date 9/12/12

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9/12/12

From: Virginia L Goodall on behalf of William E Grizzle
Sent: Wednesday, September 26, 2012 11:05 AM
To: Cynthia D Legge
Subject: FW: A-15909.2, HRPO Exempt Determination Memorandum (Proposal Log Number PC093309P1, Award Number W81XWH-10-1-0544) (UNCLASSIFIED)

From: Gaston, Sandra [mailto:SGaston@tuftsmedicalcenter.org]
Sent: Tuesday, September 25, 2012 5:44 PM
To: William E Grizzle
Subject: FW: A-15909.2, HRPO Exempt Determination Memorandum (Proposal Log Number PC093309P1, Award Number W81XWH-10-1-0544) (UNCLASSIFIED)

From: Duchesneau, Caryn L Ms CIV USA MEDCOM USAMRMC [mailto:Caryn.Duchesneau@us.army.mil]
Sent: Tue 9/25/2012 6:37 PM
To: Gaston, Sandra
Cc: Bennett, Jodi H Ms CIV USA MEDCOM USAMRMC; Katopol, Kristen R Ms CTR US USA MEDCOM USAMRMC; Strock, Abigail L Ms CIV USA MEDCOM USAMRAA; Mishra, Nrusingha C Dr CIV USA MEDCOM CDMRP; Brosch, Laura R Dr CIV USA MEDCOM USAMRMC; Duchesneau, Caryn L Ms CIV USA MEDCOM USAMRMC; Shank, Patricia A CTR US USA MEDCOM USAMRMC; Drake, Carrie E Ms CTR US USA MEDCOM USAMRMC
Subject: A-15909.2, HRPO Exempt Determination Memorandum (Proposal Log Number PC093309P1, Award Number W81XWH-10-1-0544) (UNCLASSIFIED)

Classification: **UNCLASSIFIED**
Caveats: NONE

SUBJECT: Exempt Determination for the Protocol, "Biomarkers in the Detection of Prostate Cancer in African Americans," Submitted by Sandra M. Gaston, PhD, Tufts Medical Center, Boston, Massachusetts, in Support of the Proposal, "Biomarkers in the Detection of Prostate Cancer in African Americans," Submitted by Sandra M. Gaston, PhD, Tufts Medical Center, Boston, Massachusetts, Proposal Log Number PC093309P1, Award Number W81XWH-10-1-0544, HRPO Log Number A-15909.2

1. The subject protocol and supporting documents received on 18 September 2012 in the US Army Medical Research and Materiel Command, Office of Research Protections, Human Research Protection Office (HRPO) have been reviewed for applicability of human subjects protection regulations.

2. The research involves identification of biomarker profiles that may be useful in identifying the types of aggressive prostate cancers that are more prevalent in African Americans compared to European Americans. De-identified specimens from subjects recruited at the University of Alabama at Birmingham and at the Urology Centers of Alabama will be utilized for this research.

3. The Tufts Medical Center/Tufts University Health Sciences Institutional Review Board (IRB) determined that the protocol is exempt as it is research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens if these sources are publicly available or if the information is recorded by the investigator in such a

manner that subjects cannot be identified directly or through identifiers linked to the subjects.

4. The HRPO concurs with the determination made by the Tufts Medical Center/Tufts University Health Sciences IRB in accordance with 32 CFR 219.101(b)(4). The project may proceed with no further requirement for review by the HRPO. The HRPO protocol file will be closed

5. In the event that there is a change to the subject research or statement of work (SOW), the Principal Investigator must notify the Grant Officer's Representative (GOR) and send a description of the change to the HRPO at hrpo@amedd.army.mil referencing both the proposal log number and the HRPO log number listed in the "Subject" line above. The HRPO will re-open the protocol file if necessary.

Any changes to the SOW that the GOR determines could affect the exemption status of the project, must be reviewed by the HRPO prior to approval by the Contracting Officer/Grants Officer.

6. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer/Grants Officer can authorize expenditure of funds. It is recommended that you contact the appropriate contract specialist or contracting officer regarding the expenditure of funds for your project.

7. Further information regarding this review may be obtained by contacting Patricia A. Shank, BSN, RN, CCRP, PMP, at 301-619-2282/Email: patricia.a.shank.ctr@us.army.mil.

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Note: The official copy of this memo is housed with the protocol file at the Office of Research Protections, Human Research Protection Office, 504 Scott Street, Fort Detrick, MD 21702. Signed copies will be provided upon request.

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Chapter 5
The Effects of Exosomes and Related Vesicles on Cancer Development, Progression, and Dissemination

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Abstract Exosomes are small bilayer-membrane-bound nanoparticles that are released from normal, diseased, and neoplastic cells and are present in blood and other bodily fluids. Exosomes contain a variety of signal molecules including signal peptides, mRNA, microRNA (miRNA), and lipids and have characteristic molecular features on their external surfaces (e.g., CD9 and CD81). Exosomes can function to export from cells unneeded endogenous molecules and therapeutic drugs. When exosomes and similar types of vesicles are secreted from cells and are taken up by other cells, they may act locally to provide autocrine or paracrine signals or distantly as a newly described nanoparticle-based endocrine system. Specifically, mRNA transferred to cells by exosomes can result in the production of new and novel proteins. In cancer, signals via exosomes affect the immune system via inhibition of the functions of T cells and natural killer (NK) cells, by inhibiting the differentiation of precursors to mature antigen-presenting cells, e.g., dendritic cells, and by increasing the number and/or activity of immune suppressor cells including myeloid-derived suppressor cells, T regulatory cells, and CD14, HLA-DR$^{-/low}$ cells. Exosomes from neoplastic lesions also act to provide a fertile environment in which neoplastic lesions can develop, progress, and metastasize, especially via the stimulation of angiogenesis. Exosomes have multiple potential clinical uses including the development of vaccines for targeting tumors; also, tumor-derived exosomes may be useful in diagnosis/early detection, risk assessment and prediction, in determining prognosis, and as surrogate endpoints in evaluating therapeutic and preventive approaches to cancer.

5.1 Introduction

Exosomes are small bilayer-membrane-bound nanoparticles that typically are flattened ovoid-like structures, which have been described as cup-shaped (blood) or doughnut-shaped (saliva), depending upon how and from where exosomes are

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isolated. They are released from normal and diseased cells via fusion of intracellular multivesicular bodies (MVBs) with cellular membranes and subsequent extracellular release of these nanovesicles from MVBs [1–5]. After their release from cells, a proportion of exosomes enters the lymphatic-vascular system and are transported to distant sites [6]. Thus, both normal and diseased individuals have exosomes present in their blood and other bodily fluids. Care should be taken when referring to exosomes in that the terminology, "cytoplasmic exosomes" or sometimes just "exosomes," also is used to describe the 3′–5′ exoribonuclease complex, which functions in mRNA and apoptotic DNA degradation and other pathways [7]. The 3′– 5′ exoribonuclease complex will not be discussed.

5.2 Immune Regulation by Exosomes

Exosomes are an important regulatory feature of the normal function of the immune system and in diseases in which exosomes may be involved in dysregulation of immunity [8, 9]. Many, if not all, cells of the immune system secrete exosomes including B and T lymphocytes, DCs, and mast cells [8–13]. Normally, specific forms of exosomes facilitate how antigen-presenting cells (APCs), especially DCs, present antigens to T lymphocytes and natural killer (NK) cells. For example, exosomes, which express MHC class II antigen-peptide complexes, stimulate T cells much more efficiently if the exosomes have been absorbed by functional APCs [10–13]. $CD4^+$ T cells can be stimulated to proliferate by exosomes carrying the intact stimulating antigen or the antigen-peptide in an MHC class II complex; however, the overall process of stimulating these T cells requires at least two separate subpopulations of DCs. One population of mature DCs carrying an MHC class II complex interacts with the antigen and secretes exosomes typically carrying an antigen-peptide MHC class II complex. A second CD-$8\alpha^-$, $CD80^+$, $CD86^+$ subpopulation of DCs, which may be MHC class II negative, can then interact with these exosomes to present the exosome antigen-peptide class II complexes for efficient stimulation of $CD4^+$ T cells. In addition, the activation response may be amplified by the transfer of the peptide-antigen MHC class II complexes among DCs [10]. Other pathways involving APCs and their effects on the responses of T cells to antigens are important in exosomal mediation of peripheral immune tolerance [11–14]. Similarly, MHC class I-restricted $CD8^+$ T cells are stimulated by exosomes only when the exosomes have interacted with mature DCs. Such stimulation is facilitated by adjuvant molecules including ligands for Toll-like receptors 3 and 9 [13]. Other studies have reported that exosomes from DCs can produce an antibody response and that functional B cells are needed for efficient stimulation of T cells by exosomes from DCs [14].

Exosomes participate in maternal-fetal tolerance that is important for survival of the fetus [15–18]. Specifically, the placenta releases exosomes, which contain Fas ligand (FasL), which in various models has been shown to reduce inflammation [19–22]. In addition, exosomes released from syncytiotrophoblasts contain surface ligands, i.e., ULBP1–5, to the NKG2D receptor that is found on NK, CD8, and $\gamma\delta$ T cells [15]. Release of these exosomes leads to a reduction in NKG2D receptors and

subsequent decrease in cytotoxicity of these cells in vitro [15–17]. Also, exosome-like particles have been reported to convert $CD4^+$, $CD25^-$ T cells into $CD4^+$, $CD25^+$, $Foxp3^+$ T regulatory cells (Tregs). Tregs normally suppress autoreactive T cells and hence, inhibit abnormal immune responses by their actions to prevent immune reactions to normal tissues [22].

There are numerous other interactions among cells involved in immunity via intercellular communication mediated by exosomes. For example, exosomes from mast cells can activate B and T lymphocytes and DCs. Exosomes from mast cells stimulated in vivo (mice) the maturation of DCs and facilitated their capability to present antigens to T lymphocytes. The presence of heat shock proteins (HSP) 60 and 70 in the exosomes were reported to be critical to the ability of DCs to acquire the functions necessary for antigen presentation.

Normal immune pathways may be dysregulated in autoimmune diseases. Exposure of DCs to interleukin-10 (IL-10) has been reported to increase the release of exosomes from DCs and to reduce the inflammation as well as the extent of the arthritis caused by injections of collagen [23]. Fibroblasts from patients with rheumatoid arthritis (RA) release exosomes, which contain TNFα, which can kill specific immune cells. In addition, these exosomes activate Akt via its phosphorylation causing increased phenotypic expression of NF-κB, which may increase the severity of RA [23, 24]. In addition, exosomes released from salivary glands contain autoantigens that may induce autoimmunity [25]. Exosomes also are involved in inflammation of the liver induced by fatty diets [26].

5.3 Production of Exosomes by Neoplastic Lesions

There have been many reports of exosomes being produced by a wide range of various types of tumors. Such exosomes are frequently referred to as tumor-derived (TD)-exosomes. TD-exosomes are separated primarily from the physiological fluids (e.g., urine) of patients with neoplastic lesions and spent or conditioned media from cellular cultures. Malignant lesions that have been reported to be associated with TD-exosomes include most cancers including those of the bladder, brain, breast, colorectum, kidney, lung, oral cavity, ovary, and prostate. In addition, lymphomas and melanomas have been reported to produce exosomes [8, 9].

The exosomes in the bodily fluids of patients with malignant lesions would be a combination of exosomes including those typical of normal individuals, those secondary to comorbid conditions, as well as TD-exosomes. This is the same situation that is found as to molecules circulating in blood of cancer patients in that these molecules also are from normal cells, nonneoplastic diseased cells, and tumors. Thus, the molecular characteristics of TD-exosomes used in translational research are potentially just as specific and just as sensitive as the characteristics of biomarkers in bodily fluids that are outside exosomes [8, 9, 27–29]. Actually, the molecular features of TD-exosomes may be more sensitive and specific for certain questions in translational research because selected molecules characteristic of tumors may be

more concentrated in TD-exosomes than the same molecules in the matching bodily fluids. In addition, for some malignancies, the amount of TD-exosomes correlates with the extent of the tumor.

TD-exosomes are potentially useful in translational research because they usually contain molecules characteristic of the malignant tissues from which the TD-exosomes are derived. Specifically, TD-exosomes from bodily fluids from the patients with a tumor mirror the molecular characteristics of the matching tumors [30–41]. For example, patients with melanomas have exosomes in blood, which contain Melan A/Mart 1 and other melanoma-specific molecules [3, 37]. Of interest, TD-exosomes from the blood of patients with tumors of the central nervous system (CNS) with blood-brain barriers assumed to be intact, have been reported to contain L1-NCAM/CD171, a neural molecule that was also found in TD-exosomes isolated from the matching tumors of the brain, but not in exosomes isolated from the blood from controls [30, 38–41]; however, the blood of both patients with tumors of the CNS as well as controls without tumors contained HSP70 [30, 38]. In contrast, TD-exosomes from tumors of the CNS have been reported to variably contain EGFR, EGFRvIII, HSPs27, 60, 72, 73, 80, and 90 [30, 38–41].

5.4 Nonimmune Effects of Exosomes on Tumor Progression

The growth of tumors depends upon the balance between proliferation and cell death, processes, which are regulated by multiple molecular mechanisms. TD-exosomes are involved in the growth of specific cancers via autocrine, paracrine, and endocrine regulation of the local tumor environment and hence the growth of tumors. As demonstrated in Fig. 5.1, TD-exosomes generate a fertile environment locally, supporting the growth of the primary tumor [39–46]. The enrichment of local and metastatic environments are frequently secondary to TD-exosomes whose contents are enriched in mRNAs generating proteins associated with stimulating the cell cycle of stromal cells including endothelial cells. Also, TD-exosomes may contain proteins that stimulate angiogenesis.

TD-exosomes have been demonstrated to stimulate the proliferation of endothelial cells and thus aid progression of tumors by increasing angiogenesis [39–47]. For example, paracrine stimulation by TD-exosomes from melanomas when compared with a control, i.e., autocrine exosomes derived from endothelial cells, selectively increased spheroid formation by endothelial cells and budding of spheroids as well as levels of proangiogenic cytokines including TGFβ and VEGF [42]. Similarly, Skog et al. [38] isolated microvesicles from the conditional media from short-term cultures of cells derived from human samples of glioblastomas as well as from sera from these patients and control patients. These vesicles were isolated by the typical preparations that are used to isolate exosomes, but CD9 and CD81 were not evaluated. When the source cells were examined by EM, they were described as being “covered with microvesicles” that ranged from 50 to 500 nm in diameter. Similar to “exosome” from glioblastoma patients described previously, the EGFR

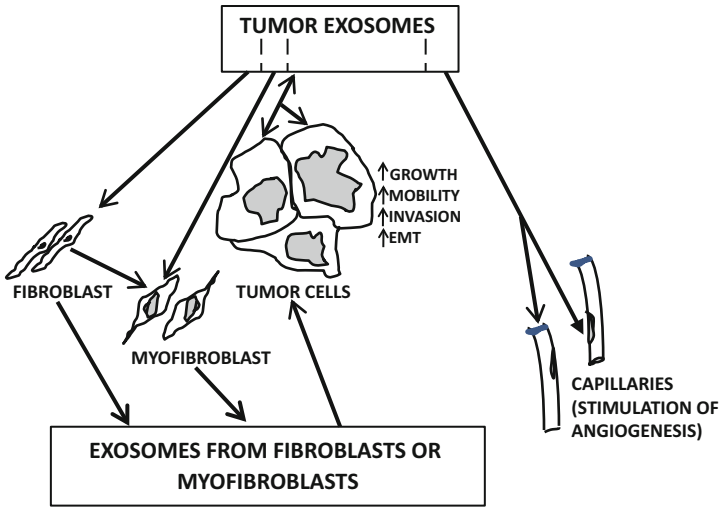

Fig. 5.1 This figure represents a model via which malignant cells interact via autocrine and paracrine activities to prepare an environment, which supports local growth of the tumor, mobility and invasion of malignant cells, induction of myofibroblasts, increased angiogenesis, and epithelial mesenchymal transition (EMT). Various responses from different compartments (subpopulations) of tumor-derived exosomes (TD-exosomes) are demonstrated by "!"

VIII receptor was found in these vesicles including vesicles from the sera of 9 out of 25 patients with glioblastomas. Also, additional mRNA mutants or variants as well as microRNAs that are typical of glioblastomas were identified in such vesicles. In these studies, they also found that microvesicles isolated by this method contained proteins that stimulate angiogenesis (e.g., angiogenin, VEGF, IL-6, and IL-8). These angiogenic proteins were enriched in the TD-vesicles compared with the contents of glioblastoma-source cells. When the TD-vesicles were internalized by microvascular endothelial cells of the brain, they stimulated an increase in tubule length that was similar to that caused by proteins that stimulate angiogenesis. The microvesicles also stimulated proliferation of the U87 glioma cell line that was derived from a glioblastoma. This suggests, in addition to stimulation of angiogenesis, autocrine stimulation by these TD-vesicles [39]. Similarly, a hypoxic environment in a primary tumor may cause the release of exosomes that are enriched in proteins that stimulate angiogenesis. This has been demonstrated with the A-431 cell line in which VEGF and other cytokine were increased by hypoxia. In addition, hypoxia reduced adhesion and increased invasion. Of note, E-cadherin was decreased and its negative regulator, snail, was increased—two proteins involved in EMT [44].

As described, TD-exosomes and related vesicles may support the growth and dissemination of tumors via providing autocrine signals to the tumor cells; however, the reports on the autocrine effects of specific cancer cell lines has been variable among different types of tumors. Specifically, TD-exosomes have been reported to increase apoptosis of well-differentiated, but not poorly differentiated pancreatic cell

lines because of the Notch pathway. Such autocrine responses would indicate that for some pancreatic tumors, TD-exosomes might inhibit local growth [48, 49]. In contrast, TD-exosomes are reported to increase proliferation via an autocrine signal in the breast cancer cell line BT-474 [50]. In addition, growth of the glioblastoma-derived cell line, U87, was increased by exosomes derived from primary tumors of the glioblastoma type. The increased growth was assumed to be due to increased proliferation; however, decreased apoptosis was not excluded [39]. Similarly, a cervical cancer cell line, C33A, incubated with exosomes from the C33A cell line transfected with the protein LAMP-1, had increased phosphorylation of Akt and ERK [51]. Of note, the activation of the Akt pathway is associated with increased proliferation and other tumor-promoting downstream molecules.

Exosomes from a gastric cancer cell line stimulated the proliferation of that cell line as well as a second gastric carcinoma cell line. This stimulation of proliferation also was via increased expression of the Akt pathway [52].

As described, tumor growth may be influenced via exosomal modulation of apoptosis. The antiapoptotic protein survivin and its associated proteins, HSP70 and HSP90, may be packaged in and on exosomes and their concentrations can be increased by cellular stresses. Of note, survivin also increases cellular proliferation and invasion, so exosomal survivin is a potent microenvironmental stimulus for the growth and dissemination of tumors [53].

Establishing a supportive environment to facilitate local growth as well as progression and dissemination of a primary tumor not only depends upon TD-exosomes, but also other microvesicles released from malignant cells as well as exosomes and other microvesicles from nonmalignant cells of the local environment of a tumor. For example, the human prostate cancer cell line (PC-3) has been reported to secrete microvesicles that are thought not to be exosomes and yet activate human fibroblasts; these microvesicles were separated after a 45-min centrifugation at 14.000 g without a nanofiltration step and without pelleting by ultracentrifugation, yet these vesicles were important in intercellular communication. The activated fibroblasts also demonstrated increased microvesicular shedding and these vesicles increased the migration and invasion of PC-3 cells. These autocrine and paracrine responses, which acted as a signal loop did not occur in the LNCaP prostate cancer cell line, which is much less metastatic. For the much more metastatic PC-3 cell line, the effects of the microvesicles on chemotaxis were attributed to CX3CL1 and its associated receptor, CX3CLR1 [43]. The differences between LNCaP and the more aggressive cell line PC-3 might be explained by the signal molecules on the surfaces of exosomes. For example, TGF-β and its associated binding complex, type III receptor betaglycan, is very strongly expressed on the surface of PC-3 cells, but not LNCaP cells [54].

TD-exosomes secreted locally may induce the activation of fibroblasts/myofibroblasts, which in turn may degrade the local extracellular matrix, increase cellular motility and invasion, and epithelial mesenchymal transitions (EMT) of neoplastic cells. Also, TD-exosomes can induce expression of α-smooth muscle actin in fibroblasts as an indication of their differentiation to myofibroblasts. Differentiation into myofibroblasts can lead to the modification of the tumor

microenvironment via the increased production of extracellular matrix such as pericellular hyaluronic acid [54]. TD-exosomes may also facilitate the growth of tumors by transfer of phenotypic properties via molecular signals to local cells, including limited oncogenic features, and may induce local cells to produce or modulate their phenotypic and molecular characteristics in order to produce phenotypic changes that support the development and dissemination of the tumor [27–29, 41, 47]. Sometimes such changes are complex, requiring sequential interactions of TD-exosomes with varying molecular characteristics [55]. In turn, local changes in the tumor microenvironment such as hypoxia, pH, cellular detachment, and stresses such as heat may affect the production, secretion, and molecular contents of exosomes [54–57].

Exosomes are efficient in transferring molecular features of cellular membranes, cytoplasm, and nuclei of the source cells to target cells and when these molecules produce oncogenic phenotypic features, TD-exosomes and/or other similar functioning vesicles have been designated as "oncosomes." For example, the variant, constitutively activated EGFR-related receptor, EGFRvIII, is oncogenic via its continual activation of the MAPK and Akt pathway and production of VEGF. Vesicles that can be obtained in vivo from glioblastoma tumors (SCID xenografts) can transfer EGFRvIII in cell culture to less aggressive glioma cells, which lack the EGFRvIII surface molecule. Once transferred, the expression of EGFRvIII remained relatively stable in these neoplastic cells so this transfer of EGFRvIII could represent a mechanism by which more aggressive phenotypes can spread horizontally among neoplastic cells. Continuing exposure of endothelial cells to EGFRvIII from exosomes also was able to transfer an EGFRvIII phenotype to endothelial cells, which stimulated their growth and hence, would stimulate angiogenesis. Note, nonneoplastic endothelial cells were not stably transformed and lost the expression of EGFRvIII when the exposure to the TD-exosomes was stopped [40, 41].

Another example of the transfer of an oncogenic phenotype by the release of vesicles from cancer cells has been described by Antonyak et al. who studied vesicles released from a breast cancer cell line (MDA-MB-231 cells) and separately from a glioma cell line (U87) that were retained on a 450 nm filter. These larger vesicles contained tissue transglutaminase (tTG) that is an oncogenic enzyme that cross-links proteins. They reported that such vesicles containing tTG plus the tTG substrate, fibronectin, could transfer to NIH/3T3 fibroblast or to human benign breast epithelial cells, MCF10A, some of the specific features of cancer cells including growth in low-serum medium and anchorage—independent growth in agar. As with other studies, some of these features could not be transferred to benign cells upon a single exposure; however, transfer of some oncogenic features to benign cells could be accomplished by continuous exposure to these vesicles [47].

5.5 Exosomes and Metastases

Metastases of tumors are controlled by many molecular pathways including genes and pathways associated with stimulating as well as inhibiting metastases [58]. In addition, metastasis of tumors is facilitated by TD-exosomes secreted from the primary

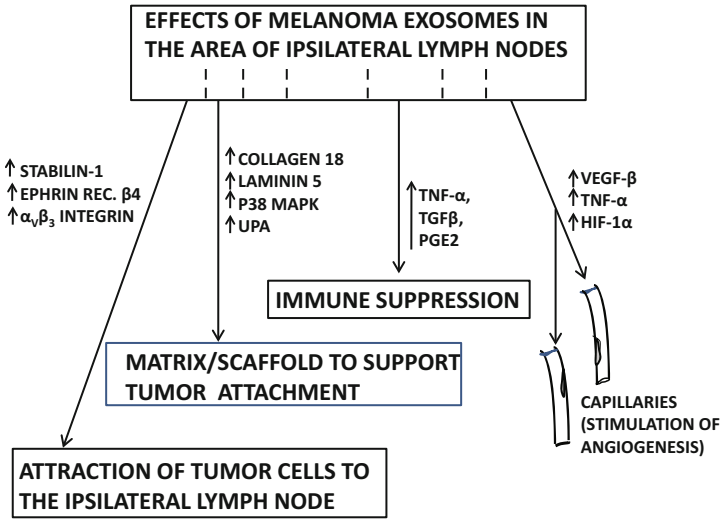

Fig. 5.2 This figure demonstrates a model of how exosomes from melanomas facilitate metastates to local ipsilateral lymph nodes via developing a fertile environment in the lymph node. Data are based on analysis at the mRNA level as adapted from Ref. [81]. The potential variability of TD-exosomes is emphasized in the cartoon by different compartments or subpopulations of TD-exosomes. *HIF* hypoxia induced factor, *MAPK* map kinase, *REC* receptor, *TNF* tumor necrosis factor, *TGF β* transforming growth factor β, *PGE2* prostaglandin E2, *VEGF* vascular endothelial growth factor

tumor. These TD-exosomes can induce factors at the metastatic site, which attract tumors, build scaffolds for the attachment of metastatic cells, aid in the survival of metastatic tumor cells, suppress immunity, and stimulate angiogenesis [42–50, 59].

Exosomes from melanomas have been reported to travel to sentinel lymph nodes and aid in establishing an environment favorable for metastases. Melanoma cells were reported to be recruited to the sentinel lymph node by exosomal stimulated production of stabilin 1, ephrin receptor β4, and integrin $\alpha_v\beta_3$. The matrix to support the growth of melanoma cells also was found to be modulated by exosomes via the increased expression of collagen 18, laminin 5, map kinase (p38), and urokinase plasminogen activator protease. In addition, the exosomes stimulated angiogenesis by the production of vascular endothelial growth factor β (VEGFβ), TNFα, and hypoxia inducible factor 1α (HIF-1α). As in Fig. 5.2, this molecular priming of the lymph node ipsilateral to the primary site of the melanoma causes selective metastases to the ipsilateral lymph node [59]. In addition, exosomes induce local changes in melanoma that may aid in dissemination [42].

In a rat model of pancreatic cancer, conditioned media from a cell line of a syngenic tumor was separated into a soluble and exosomal fraction. The exosomal fraction was most effective in supporting metastatic spread to lymph nodes and the lung; however, the combination of soluble plus exosomal fractions were much more efficient at supporting these metastases. This effect was greatly reduced in cell lines in which there was knockdown of splice variants of CD44, especially CD44v6, on which

the induction of an extracellular matrix was reported to depend; the extracellular matrix contained c-Met and urokinase-type plasminogen activator receptor. Other exosomes from pancreatic tumors independent of their aggressiveness could interact with the soluble matrix to facilitate tumor attachment and subsequent growth [55].

Many of these studies have been based upon the responses of cells to TD-exosomes or other vesicles isolated from culture media of cell lines. Much more work is required to characterize autocrine, paracrine, and endocrine interactions of exosomes in the growth, progression, and metastases of tumors in vivo.

5.6 Effects of Exosomes on Immune Surveillance of Neoplasia

As discussed, the immune system should recognize the development, progression, and dissemination of neoplastic lesions and should theoretically inhibit the growth of such lesions via cellular and other immune reactions. Unexpectedly, the presence of tumors actually suppresses immunity permitting the more rapid growth of tumors [60–62]. This, plus the involvement of exosomes in immune regulation, suggested to investigators that neoplastic lesions might release TD-exosomes, which could provide signals to inhibit immunity. Thus, it was hypothesized that signals provided via TD-exosomes were involved in the partial suppression of immune surveillance and that this facilitated the progression of neoplasia. This potential mechanism is supported by our report that transplanted tumors demonstrated increased growth in mice after injection of exosomes isolated from the same tumors [61]. In subsequent studies, it has been shown that TD-exosomes act to suppress immune surveillance via multiple pathways and multiple phenotypic effects. These include reduced cytotoxicity and decreased interleukin-2- mediated proliferation of NK and T cells; APCs are decreased by TD-exosomal signals and cells that suppress immunity are increased [60–67]. Specifically, the observed changes in the function of NK cells caused by TD-exosomes is associated with decreased release of perforin, inhibition of Jak-3, and decreased expression of cyclin D3 [61]. Based upon these other studies, it has been concluded that the decrease in immune surveillance induced by tumors is, in part, caused by the release of exosomes [8, 9, 19, 61–73].

The molecular signals provided by TD-exosomes to immune cells are likely to be diverse among the various categories of tumors. For example, ovarian epithelial and oral neoplastic lesions have been reported to secrete exosomes that contain FasL, TRAIL, and/or related molecules (e.g., TNFα) via the TRAIL/FasL pathways that can target and cause apoptotic cellular death of activated T cells [19, 21, 24, 63]. Specifically, TRAIL or FasL can suppress CD-3ζ and Jak-3 in activated T lymphocytes and induce apoptosis [63]. However, in our prior study of the effects of TD-exosomes on the in vivo growth of matching tumors, the TD-exosomes contained neither TRAIL nor Fas ligand [61]. In addition, TD–exosomes, which contain TGF-β1 down regulate the expression of the NKG2D receptor, which is a receptor involved in activation of $CD8^{+}$T and NK cells [64]. These observations support the concept that TD-exosomes may inhibit immune surveillance via multiple pathways,

which directly inhibit the ability of T and NK cells to target and destroy neoplastic cells.

Exosomes also inhibit the immune system and increase immune tolerance by reducing the activity and/or numbers of APCs, especially dendritic cells [65–70]. Normally, exosomes from DCs are necessary to stimulate T and NK cells to kill tumor cells and hence APCs should aid in the reduction of the growth of tumors [65–73]. However, TD-exosomes inhibit immune surveillance by reducing the number of mature DCs in a time- and dose-dependent manner. This occurs via signals from exosomes, which increase the phenotypic expression in DCs of IL-6 and the phosphorylation of Stat 3 [70]. TD-exosomes also directly reduce the numbers of APCs via inhibiting the differentiation of $CD14^+$ monocytes to mature APCs. This inhibition results in a shift of $CD14^+$ cells to an immunosuppressive subset of $CD14^+$HLA-$DR^{-/low}$ cells, which may secrete TGFβ and thus directly inhibit T cells [68, 69].

In addition to the direct targeting of T and NK cells and the indirect effects on immunity via reducing the number and effectiveness of APCs, TD-exosomes may cause the suppression of the immune system by increasing subpopulations of cells, which inhibit immunity such as $CD11b^+Gr\text{-}1^+$, myeloid-derived suppressor cells (MDSCs) [74–82]. MDSCs have been noted to be increased in tumors as well as in blood and spleens of mice with transplanted syngenic tumors and MDSCs can be decreased in the blood and spleens of these mice by removing the implanted tumors [75–77]. Of note, increased numbers of MDSCs in humans with specific tumors have been associated with increased progression of tumors and decreased patient survival [76–79]. This may be via the action of MDSCs to inhibit $CD4^+$ and $CD8^+$ lymphocytes as well as NK cells; the direct contact of MDSCs with NK cells decreases activation of NK cells [74, 75, 83]. The release from tumor cells of soluble molecular mediators such as GM-CSF, in addition to exosomes, may also increase the number of MDSCs. TD-exosomes that contain TGFβ and prostaglandin E2 can increase MDSCs [78, 80]. Also, exosomes with HSP72 may modulate receptors to MyD88 as well as Toll-like receptor2 of MDSCs, causing MDSCs to be activated by increases in IL-6 and Stat 3 phosphorylation [80]. Of note, the study of the interaction of TD-exosomes with TLR pathways and MyD88 should be studied more extensively in cells isolated from in vivo tumors because of phenotypic changes in cells induced by long-term cultures [8, 81, 83].

Immune surveillance of tumors also can be reduced by TD-exosomes, which can cause $CD4^+$, $CD25^-$ T cells to differentiate into $CD4^+$, $CD25^+$, $Foxp3^+$ Tregs via signals resulting in the phosphorylation of SMAD2/3 and Stat3 [82].

In summary, immune surveillance is reduced and avoided by tumors because TD-exosomes can inhibit the activity of T and NK cells, reduce the maturation of precursor cells to APCs especially dendritic cells and thus indirectly inhibit the functions of APCs, as well as increase cellular subpopulations, which suppresses immunity including myeloid-derived suppressor cells, Treg cells, and $CD14^+$-HLA-$DR^{-/low}$ cells. The effects of TD-exosomes on immunity are demonstrated in Fig. 5.3.

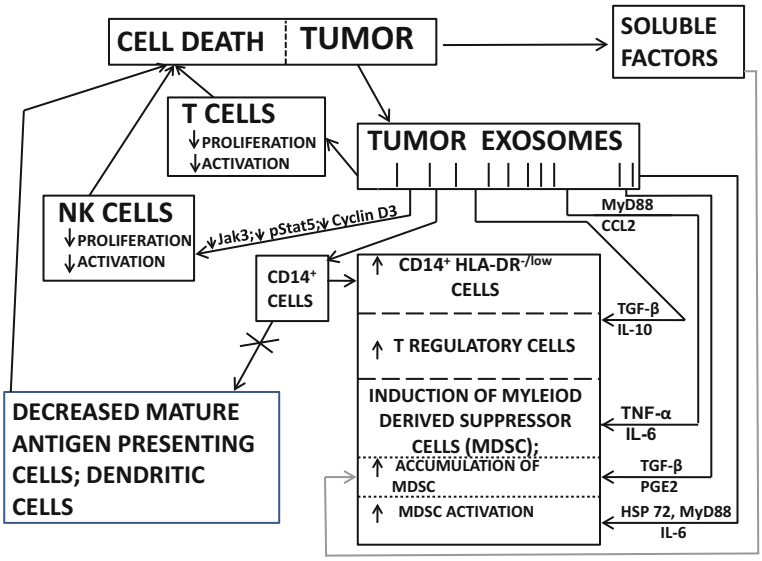

Fig. 5.3 This figure demonstrates a murine model of the multiple actions of TD-exosomes released from neoplastic cells to suppress immune surveillance. TD-exosomes reduce the number of antigen-presenting cells (e.g., blocking the differentiation of myeloid precursor cells into dendritic cells) as well as the activity and/or proliferation of NK and T cells. In addition, cells, which suppress the immune system, including Treg cells, MDSCs, and $CD14^{+}$, HLA-$DR^{-/low}$ cells are increased in number, distribution, and/or activity via the actions of TD-exosomes. Overall, these actions reduce the cytotoxicity of the immune system against malignant cells. In this cartoon, the variability of TD-exosomes is emphasized by the individual compartments or subpopulations of the TD-exosomes, each of which produces different effects on the immune system. *MDSC* myeloid-derived suppressor cells, *NK* natural killer, *Treg* T regulatory cell, *HSP* heat shock protein, *PGE* prostaglandin E, *IL* interleukin, *TGF* transforming growth factor, *TNF* tumor necrosis factor

5.7 Effects of TD-Exosomes on the Resistance of Tumors to Therapy

Exosomes provide a pathway via which unneeded and other molecules are exported from cells [2, 8, 9, 84–87]. Specific chemotherapeutic and other drugs may be eliminated via this pathway and TD-exosomes may be important to the development and maintenance of chemoresistance [8, 9, 85–88]. Doxorubicin and cis-platinum, but not 5-fluorouracil, are efficiently exported by TD-exosomes and thus TD-exosomes may provide a pathway of chemoresistance of specific malignant cells [86–87]. Shedden et al. [86] combined genes associated with the shedding of exosomes into a "vesicle shedding index," which in the "NCI 60-cell-line panel" positively correlated with the 50 % growth inhibition (GI_{50}) for most of the 171 compounds of the NCI "Standard Anticancer Agent Database." Also, when the actual shedding of vesicles from six cell lines were measured, the shedding rate correlated positively with resistance to

doxorubicin, a drug that is exported in vesicles; however, there was not a correlation with resistance to the drug, 5-fluorouracil, which is not efficiently exported in vesicles [86]. The export of drugs such as doxorubicin is thought to involve the vacuolar protein sorting 4a (VPS4a), which is a protein essential for the formation of multivesicular bodies and thus the secretion of exosomes. Specifically, in a model using an erythroleukemic cell line, K562, disruption of the VP54a pathway inhibited the formation of exosomes and the efflux of doxorubicin [88].

5.8 The Uses of Exosomes in Detection, Diagnosis, Prognosis, Prediction, and Treatment of Neoplasia

TD-exosomes affect many aspects of the development, progression, and dissemination of their neoplastic cells of origin, typically mirroring the molecular features of their originating tumors. TD-exosomes may contain enriched concentrations of biomarkers, hence molecular assays of TD-exosomal preparations may be useful in translational research and/or in making clinical decisions. Uses include early detection, diagnosis, and risk assessment as well as in prediction of therapeutic efficacy, in determining prognosis, and as surrogate endpoints in evaluating chemotherapeutic and novel therapies as well as approaches to prevention of neoplasia [27–29]. Of importance in translational research is that exosomes can be picked up and carried in blood as well as shed into multiple biological fluids such as urine, ascites, and pleural fluids, biological fluids that can be obtained easily for clinical uses. The measurements of biomarkers in the exosomal compartments of biological fluids are likely to be just as sensitive and specific as measurements of the same biomarkers in the matching fluids [8, 9, 27–29]. For specific biomarkers, which may be even more concentrated in exosomes than in the biofluid from which exosomes have been removed, the measurement of a biomarker or biomarker panel in the exosomal fraction may be more sensitive and specific for answering specific clinical questions [8, 9, 27–29]. This must be determined for each clinical use by translational research for each separate biomarker or biomarker panel [27–29]. Such multiple uses have been proposed [89–98]. For example, Claudin 4, a biomarker of ovarian epithelial tumors, was increased in exosomes separated from the blood of patients with ovarian carcinoma [89]. MicroRNAs that have been proposed to be useful in the diagnosis of lung cancer have been found in TD-exosomes from patients with lung cancer [90, 91]. Similarly, δ-catenin, PCA-3, and TMPRSS2:ERG, which are putative molecular biomarkers useful in the early diagnosis of prostate cancer, have been detected in TD-exosomes in urine of patients with prostatic cancer [92–95]; such biomarkers in exosomes also may be useful in determining therapeutic responses. Similarly, the presence in biological fluids of TD-exosomes containing biomarkers characteristic of tumors of the bladder, brain, colorectum, and other tumors such as melanomas and renal cell carcinomas have been reported [3, 6, 30, 37, 39, 96–101]. Thus, the presence of increased molecular biomarkers in the biological fluids from individuals may be useful in clinical decisions as well as in translational research based on measurements of specific biomarkers.

As TD-exosomes may contain increased concentrations of specific tumor antigens, exosomes could be used in stimulating cytotoxic T-lymphocyte responses (CTL). Specifically, TD-exosomes could be used to stimulate DCs [3–5] and the in vitro generation by the DCs of exosomes could avoid the negative therapeutic effects of the use of TD-exosomes (e.g., inhibition of immune surveillance). To date, trials with this approach have been safe, but responses have been only modest [57, 62, 63, 67, 102].

Alternatively, due to the actions of TD-exosomes in suppressing immunity in patients with neoplastic lesions, the direct targeting of TD-exosomes could be used to reduce the suppression of the immune system in patients with tumors. Several strategies have been proposed to reduce the inhibitory effects of TD-exosomes on immune surveillance in patients with cancer. One approach would be to prevent or reduce the transfer into the TD-exosomes of molecules, which may suppress immunity such as FasL. Alternatively, molecules, which might selectively stimulate immune cells could be introduced into TD-exosomes in vitro. This would permit targeting tumors selectively [8, 9]. In addition, factors that decrease the release of TD-exosomes such as cellular pH might be utilized [56, 103] as could methods, which can specifically remove TD-exosomes from the circulation. For example, TD-exosomes might be removed from blood using some form of "hemopurifier" that selectively removes TD-exosomes via fixed antibodies that specifically bind TD-exosomes and thus, reduce the inhibitory effects of exosomes on immune surveillance [8, 9].

Exosomes have been reported to function in selectively exporting specific drugs (e.g., doxorubicin and cisplatinum) from neoplastic cells [86, 87]. Thus, TD-exosomes involved in exporting drugs from cells might be targeted to make specific drugs more efficacious. Such targeting to decrease the removal of therapeutic drugs via exosomes might be via reducing the production or release of TD-exosomes from neoplastic cells. This might be accomplished via local changes in pH or by selectively targeting the VPS4a signaling pathway [56, 86–88, 103].

As TD-exosomes can selectively target malignant cells via autocrine interactions, they could serve as a method of delivery of drugs, small molecules, and agents of gene therapy to selected tumors. For example, curcumin, a polyphenol with antiinflammatory and antitumor activities, has been used to reduce the effects of TD-exosomes in inhibiting NK cytotoxicity [104]. Unfortunately, solubility and bioavailability has limited the effectiveness of curcumin therapeutically, although its usefulness can be improved by its delivery via encapsulation in lipid preparations such as liposomes [105]. In comparison, the delivery and bioavailability of curcumin can be improved greatly by the incorporation of curcumin into exosomes. Delivery of curcumin by exosomes was found to be much more effective than delivery by liposomes in preventing septic shock in a murine model [106]. While not directly related to cancer therapeutics, this approach shows how specific drugs could be targeted to neoplastic cells by exosomes.

Developments in this area are being advanced by several commercial as well as academic organizations. The potential use of exosomes in clinical medicine has been the focus of many patents including the uses of exosomes to increase immune reactions to tumors, to facilitate the specific delivery of therapeutic molecules to tumors,

to block or reduce the export of drug and molecules utilized in cancer therapy, and to remove molecules by "hemopurifier". This work is being performed on exosomal as well as nonexosomal vesicles. For example, vesicles from lower organisms such as ameba are being proposed as vehicles to transfer a wide range of molecules to eukaryocytic cells (e.g., patent application to I. Tatischeff et al.). In addition, vesicles have been constructed from erythrocytic ghosts and these vesicles were filled with doxorubicin coupled to folate. The preparation was stable and demonstrated increased cytotoxicity compared with doxorubicin or doxorubicin-loaded vesicles [107]. Of interest, mesenchymal stem cells have been selectively immortalized by a lentiviral transfection of the myc gene. The purpose of this cell line was to provide a stable, uniform source of exosomes for potential therapeutic uses [108]. Thus, approaches using vesicles for the selective delivery of drugs, bioactive molecules, preventive agents, and gene therapy are in active development.

5.8.1 Exosomes should be Defined or Subclassified Based on the Combination of Size and their Biological Properties

Lastly, we would like to express our concerns as to the current terminology and "definitions" used in the field of exosomes and the general field of extracellular vesicles. Why not extend the definition of exosomes into nanoparticles of various sizes instead of restricting their size to 30–100 nm? Most bodily fluids, including blood (serum and plasma), breast milk, saliva, pleural fluid, amniotic fluid, ascites, urine, cerebrospinal fluid, semen, bronchoalveolar lavage fluid, and synovial fluid contain exosomes [99–101] and other membrane-bound vesicles. Exosomes may have different molecular, morphological, and biophysical properties based on the tissue or biofluid from which they are isolated. For example, the exosome-like particles from semen were reported to be round to egg-shaped, to contain internal vesicles and to range in size from 50 to 500 nm [100]; however, exosomes isolated from ascites were noted to be almost identical in size (30–100 nm) and ultrastructure to exosomes from matching samples of blood [101]. Exosomes isolated from saliva were described as < 100 nm in diameter and doughnut-shaped with a height to width ratio of about 0.04 [34]. The vesicles recovered from cell culture media have been reported to be larger and in some cases their sizes have been described as biphasic, with most being 100–200 nm and 400–1,000 nm [86] in largest dimension.

The "sizes" of exosomes become an important issue based on past and current studies, which have used the terms "microvesicles," "oncosomes," or "exosomal-like particles" instead of exosomes to include together with exosomes, vesicles that bud directly from the surface lipid rafts of cellular membranes without involvement of MVBs [40–50, 109]. Usually, such studies vary as to how membrane-bound particles are separated from fluids, especially from spent or conditioned media from cell cultures, as well as the surface biomarkers that are used to identify subgroups of "vesicles." One possibility that may explain the larger vesicles found in spent media is that cells in two-dimensional (2D) cultures produce exosomes with different sizes,

functions, molecular contents, and/or molecular surface markers than classic "exosomes" isolated from blood. For example, interactions of cell lines of tumors grown in contact with other cells (leukocytes) may secrete exosomes with different contents and functions than cells grown in isolated 2D culture environments [83, 110]. Similarly, exosomes obtained from short-term cultures of cells derived from xenografts function differently than exosomes from cells maintained in longer term cultures [83]. In addition, the actual biological functions of extracellular vesicles that do not meet the typical definition of exosomes may have similar functions in general intercellular communications. If the basic structures and functions of particles are the same, an arbitrary size separation may not be that important. Similarly, using a group of surface biomarkers to define the exosome subcomparment in some cases may be too restrictive in that the exact surface markers may vary, depending upon the specific physiological actions of exosomes (e.g., a different surface marker or lack of a surface marker might target exosomes to different subgroups of cells). Also, even a preparation used in isolation of vesicles that meets the standards of a classically defined exosomal preparation (e.g., CD9 and CD81 molecules on Western blots) does not ensure that all of the membrane-bound particles in that preparation meet the standard molecular characteristics of "exosomes." In addition, just because most of the vesicles in an exosomal preparation meet typical definitions of an exosome, does not mean that those exosomes creating the observed responses are from the subpopulation expressing classical exosomal markers. It is likely that each cell produces many different subpopulations of exosomes as well as different, nonexosomal vesicles; thus, one cell may provide multiple signals to different cellular groups both locally and distantly. Each population of exosomes secreted by one cell type would likely contain multiple distinct subgroups of exosomes/vesicles based both on their surface molecules (interaction signals) and molecular contents (cellular modulation signals). In addition, the sizes of exosomes may be dynamically changed over time from the point of initial release to their reaching their final destination. To test this hypothesis, we need to develop in vivo imaging technology to keep trafficking of an individual exosomes.

A major problem in the analysis of exosomal functions is that one must read carefully the details of the methods of isolation of each study in order to understand which subgroups of membrane-bound vesicles are being studied; this includes understanding their biochemical and physiological characteristics and functions and how studies of specific vesicles relate to those of classic exosomes. Specifically, some studies use a 200–220 nm filter to exclude larger particles from a preparation; alternatively, this filter or a larger pore filter (450 nm) may be used to retain the particles to be evaluated. Similarly, it is frequently unclear as to how the preparations are tested or not tested as to the expression of surface markers such as tetraspanins (e.g., especially CD9 and CD81). Publications are becoming chaotic as to studies of exosomes or related vesicles and must in the future be clearer as to which subpopulations of "vesicles" are being evaluated and how experimental results relate to those elicited by classic exosomal preparations. Unfortunately, methods are not always clear or the methods may be relegated to supplementary information and thus, are not an integral component of the manuscript. Even manuscripts, which indicate that the "vesicles"

studied are similar to other studies may have studied different vesicles. Specifically, studies of "exosomes" may refer to the results of manuscripts that were focused on different types of "vesicles" and indicate that these prior results were obtained from "exosomes." The molecular, functional, and morphological characteristics of different subgroups of vesicular nanoparticles secreted/excreted by cells are an area that should be clarified by future research.

Specifically, molecules on the surface of exosomes are frequently used to define and to demonstrate that exosomal preparations contain isolated exosomes [37, 111–116]. These include selected tetraspanins, CD-9, CD-49d, CD-63, CD-81, and CD-82, which have been described as markers of the external surfaces of exosomes. Other molecules proposed to be characteristic of the surface molecules of exosomes include the integrin $\alpha_v\beta_3$, (CD51, CD61), transferin receptor (CD71), Alix, CD80, CD86, externalized phosphatidylserine, milk fat globule-E8/lactadhern (MFG-E8), ICAM-1, CD-96, Rab-5b, and MHC class I and II complexes . Exosomes secreted by specific types of tumors such as melanomas may express specific molecules such as caveolin-1 [3–5, 8, 9, 37, 115] while other molecules, e.g., CD11a and its ligand, CD54 and CD11b, are more specific for inflammatory cells. In addition, even the tetraspanins may not always be expressed on every type of exosome; however, the surface molecules used most commonly to define exosomes from all cell types include CD9 and CD81.

Similar to nanoparticles, the sizes of exosomes may also have effect on exosomes trafficking. The exosomes circulating in blood and/or present in some bodily fluids may be influenced by the sizes of vesicles initially released from cells, in that smaller exosomes may penetrate the spaces of lymphatics and capillaries more readily and selectively than larger exosomes/vesicles; hence, smaller exosomes may be represented more extensively in blood. It remains to be determined if smaller exosomes actually penetrate more readily into the vascular-lymphatic system or into specific cells and/or if larger exosomes remaining in the interstitial space might be more important in modulating local cellular functions via autocrine, paracrine, and "other-crine" interactions.

5.9 Summary

Exosomes are an important component of a newly identified method of local (autocrine, paracrine, and other-crine) and distant (endocrine) intra- and intercellular communication that functions in normal cells. Tumors have adapted or hijacked this approach to intercellular communication to aid in their growth, progression, and dissemination by signals, which aid in the production of a fertile environment of the primary tumor as well as the potential metastatic sites of tumors. TD-exosomes also inhibit the immune system in humans and other animals with tumors and TD-exosomes facilitate the resistance of neoplasia to specific drugs by exporting selective chemotherapeutic agents and other potentially therapeutic molecules from cells.

Thus, the clinical impact of TD-exosomes is that TD-exosomes aid tumors in avoiding immune surveillance, in aiding neoplastic lesions to progress and disseminate more rapidly, and as a pathway of therapeutic resistance. Because of the deleterious effects of TD-exosomes in blood and other bodily fluids, TD-exosomes might be selective targets for therapeutic interventions via their removal; exosomes and other vesicles might, in contrast, serve as vehicles for selective delivery of drugs, small molecules, preventive agents, or agents used in gene therapy to neoplastic lesions. Additional clinical uses might focus on using TD-exosomes as a subcompartment of biological fluids in which biomarkers are measured to aid in the early detection and diagnosis of diseases for determining prognosis, for prediction of therapeutic efficacy, and to measure therapeutic responses to therapy. Such uses would rely on the molecular contents of TD-exosomes, which should be different than exosomes from either associated diseased controls or from normal individuals. All these potential uses of exosomes are currently active areas of translational research.

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Post-transcriptional processing of genetic information and its relation to cancer

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Abstract

During the development, progression and dissemination of neoplastic lesions, cancer cells hijack normal pathways and mechanisms, especially those involved in repair and embryologic development. These pathways include those involved in intercellular communication, control of transcription, post-transcriptional regulation of protein production including translation of mRNAs, post-translational protein modifications, e.g., acetylation of proteins, and protein degradation. Small, non-translatable RNAs, especially microRNAs (miRs), are Important components of post-transcriptional control. MiRs are produced from areas of the genome that are not translated into proteins, but may be co-regulated with their associated genes. MiRs bind to the 3′ untranslated regions of mRNAs and regulate the expression of genes in most cases by either promoting the degradation of mRNA and/or inhibiting the translation of mRNAs into proteins; thus, miRs usually cause a decrease in protein levels that would be expected if the mRNAs were translated normally. It is early in our understanding of how miRs affect neoplastic processes, but miRs are expressed differentially in most cancers and have been associated with tumor progression, chemoresistance and metastasis. MiRs are present in nanovesicles, such as exosomes, and thus are likely involved in intercellular communication, especially in neoplasia. MiRs are attractive targets for novel therapies of cancer as well as potential biomarkers that might be useful for early detection and diagnosis, and for prediction of therapeutic efficacy. MiRs also could aid and in determining prognosis, evaluating novel therapies, and developing preventive strategies by their use as surrogate end points.

Key words: biomarkers, mRNA, microRNA, post-transcriptional regulation, transcriptional regulation, translation

Transcription is the process by which sequences of the genome are read by the enzyme RNA polymerase II and pre-forms of messenger ribonucleic acid (mRNA) are produced. These pre-forms of mRNA (pre-mRNA) are converted into mRNA and the mRNAs subsequently are translated into proteins by ribosomes that match the sequences of the mRNA with triplet codes on transfer RNAs (tRNAs), each of which carries a unique amino acid. The complex of ribosomes, mRNA and tRNAs translate mRNA into amino acids that are bound together in a growing polypeptide chain ultimately to become a protein that matches the mRNA. Based on studying the genomics of prokaryotes, the concept arose that "one gene" was translated into "one protein"; however, based on the study of eukaryotic genomics, it is recognized that one gene ultimately can generate many different proteins. Therefore, fewer than 40,000 different genes may produce hundreds of thousands of unique proteins. In general, proteins generate the phenotypes of normal and diseased cells and tissues.

Because each cell type has specific roles and functions at multiple levels, i.e., organism, tissue and cell, the abundance, variety and type of

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proteins in each cell type may vary and may be cell-specific. Thus, a hepatocyte of the liver must be able to run the metabolic machinery necessary to produce glucose by gluconeogenesis and also produce and secrete many different proteins, e.g., albumin. In addition, the liver converts some waste products into bile. By contrast, skeletal muscle cells use glucose to power contractile proteins so that these cells can change size and shape rapidly. The cells of skeletal muscle do not produce glucose, albumin or bile. Because the DNA code is the same in each cell of an organism, accurate maintenance of the selective phenotypic characteristics in different types of cells is accomplished by genetic and epigenetic control of transcription, post-transcriptional processing including control of translation, and post-translational modifications of proteins.

In general, the mRNA produced from DNA in prokaryotic cells reproduces the base pattern of the complementary strand of the DNA. In eukaryotic cells, the DNA of a gene is separated into transcribed exons and untranscribed introns, i.e., the codes of introns are not incorporated into the final mRNA. Introns and other untranscribed areas of DNA may control the transcription of the gene or produce small RNAs that may regulate mRNA. In eukaryotic cells, a precursor form of mRNA (pre-RNA), which includes the RNA codes of introns and exons, initially is transcribed from the DNA, then the pre-mRNA is edited to remove the regions of the mRNA that correspond to the introns. During processing of pre-mRNA, specific exons may or may not be included in the final mRNAs, which results in "splice variants" of proteins. Similarly, recent studies have shown that there may be additional editing of mRNAs by unknown mechanisms so that some mRNAs may not mirror the DNAs from which they were originally transcribed (Li et al. 2011); however, these results are controversial.

Post-transcriptional regulation

After transcription, many processes (Table 1) may occur to produce the large number of proteins that make up both the cellular and extracellular components of a tissue, hence its phenotype. These processes produce many changes in the proteins that would not be predicted based on the code and/or structure of the DNA. Of the post-transcriptional processes, we focus here on how small RNAs regulate mRNA, therefore the amount of proteins produced from specific genes.

MicroRNAs (miRs)

MiRs are short (hence the name), approximately 22 nucleotide forms of RNA that are single-stranded; they are not translated into proteins. By affecting the degradation and translation of mRNA, however, miRs can modulate levels of proteins.

For convenience, microRNA usually is abbreviated miR. We will use miR for this class of molecules and mir as a prefix to denote specific human precursor forms of miRs (pre-miRs), which ultimately may be processed into the same mature miRs; thus an example of a mature miR is miR-let7 while an immature form would be mir-let7. To date, miRs have been described in most cells except some types of plants, e.g., certain marine plants and some fungi (Lee et al. 2010). The functions of miRs may vary in plants and lower organisms; however, we will focus primarily on the importance of miRs in mammals.

MiRs were discovered in the worm, *C. elegans*. In this worm, the extent of the expression of the heterochronic gene, lin-14, that regulates developmental timing critical for larval transition (Chalfie et al. 1981) occurs by the complementary binding of a small RNA, lin-4, to the 3′ untranslated region

Table 1. Post-transcriptional regulation in eukaryotes

Editing of the pre-mRNA
Alternate splicing of the pre-mRNA to include or exclude specific exons
Editing of the mRNA by enzymes, e.g., adenosine, to inosine
Editing of the mRNA by undefined mechanisms, changing the code of the mRNA and ultimately the structure of the proteins expected based on the DNA
Modulation of movement of mRNA out of the nucleus
Modification of the mRNA by small forms of RNA, including miRs
Degradation of the mRNA including control of degradation by miRs
Incorporation of atypical coded bases in tRNA, e.g., inosine, and their exit from the nucleus
Control of translation of mRNA to protein by miRs and by ribosomes, e.g., protein interactions with the internal ribosomal entry site (IRES)
Primary post-translational modifications of the initial proteins
Control of proteins by degradation

(3′-UTR) of the lin-14 mRNA (Lee et al. 1993, Wightman et al. 1993). Subsequently, a small non-coding RNA, let-7, in *C. elegans,* was identified as a critical regulator of cellular development, which suggests that these miRs may act as fundamental developmental regulators (Reinhart et al. 2000). Ultimately, RNA molecules of the let-7 type were found to be conserved in many species including humans. With this observation, it became apparent that regulatory small RNAs were a general biological mechanism for post-transcriptional control of genetic information. MiRs function in the normal development and growth of cells from plants to man. Most miRs target developmental processes that involve cellular control, proliferation, and cell death. By contrast, processes that characteristically involve routine maintenance functions common to all cells typically are not controlled by miRs. At present, it is believed that about 1/3 of the human genome may be under transcriptional regulation by miRs (Chen 2005, Phillips 2008, McDaneld 2009).

Production of miRs

Typically, the genes that produce miRs can be located in an anti-sense orientation to exons or introns or in areas of DNA that were thought to be non-coding. Nevertheless, the genes that produce miRs are regulated by promoters and other regulatory mechanisms. Also, some genes for miRs may be in a sense orientation within introns or other non-coding areas of the DNA. This orientation within genes permits a miR to be co-regulated with its "related gene." For example, a genetic sequence that codes for a specific miR, e.g., microRNA-xxy, which regulates the mRNA transcribed from gene y is located within the intron between exons 4 and 5 of the gene for y. Thus, as gene y is transcribed, the miR that binds to the mRNA of gene y would be transcribed with y and would be regulated, in part, by factors that control the transcription of y.

MiRs usually are produced from specific genes by RNA polymerase II (less commonly by RNA polymerase III) as a "primary miR" (pri-miR) that contains hundreds of nucleotides and a poly A tail at the 3′ end. When pri-miR is from a transcribed gene, pri-miR may be separated in the course of splicing, e.g., removing the intron areas of the mRNA. RNase III enzymes, Drosha and Pasha, enzymatically generate from the pri-miR approximately a 70 base form of RNA designated pre-miR, which leaves the nucleus as a complex with Exportin-5 (Chen 2005, Phillips 2008, McDaneld 2009). The structure of the pri- and pre- forms of miR, though short, includes a hairpin loop. Once processed to miR, the hairpin loop is removed. The pre-miR then is cleaved by an RNA III enzyme, dicer, and the cleaved form of RNA is incorporated into an Argonaute-protein-containing complex called an RNA induced silencing complex or RISC. When bound as part of the RISC, the RNA is composed of two complementary strands. One strand then is cleaved, released by the RISC and degraded. The RISC orients the remaining strand (now designated a mature miR) so that it can bind optimally to target areas of mRNAs (Chen 2005, Phillips 2008, McDaneld 2009).

Functions of miRs

The target areas of miRs in most cases are specific sequences of the 3′-UTR of the mRNA. The same RISC-miR complex can bind and regulate many different mRNAs if they have the same or similar sequences in their 3′-UTRs so that the same miR can modulate concomitantly many different mRNAs (e.g., 100) (Chen 2005, Phillips 2008, McDaneld 2009).

If there is a strong complementary Watson-Crick match with the bases of the target region of a mRNA, the mRNA is cleaved by an energy requiring (ATP) process so that the poly A end of the 3′ mRNA and the capped 5′ end of the RNA are removed, which enables rapid mRNA degradation of each fragment of the mRNA by exonucleases (Chen 2005, Phillips 2008, McDaneld 2009). The RISC and its miR are stable and continue to be biologically active so it can bind other mRNA molecules (Chen 2005, Phillips 2008, McDaneld 2009). Unlike mRNAs, mature miRs are thought to be very stable in vitro as well as in vivo; in addition, miRs can be identified in fixed and paraffin embedded tissues so archival paraffin blocks are used for their analysis (Bovell et al. 2012).

If the miR does not have a strong base pairing with a sequence of the 3′-UTR, it still may bind, but less avidly, to the target mRNA. In such cases, the binding may not result in cleavage of the mRNA, but the bound miR inhibits the translation of the mRNA and sets up the mRNA for eventual degradation by the transfer of the mRNA to processing bodies or "P-bodies," the sites where most mRNAs, whether regulated by miRs or not, are destroyed or are stored prior to degradation (Chen 2005, Phillips 2008, McDaneld 2009). MiRs also may act in other ways to affect genetic information. For example, they may bind to regulatory introns to modulate transcription and/or miRs may inhibit

translation of mRNAs (Chen 2005, Phillips 2008, McDaneld 2009).

It is important to understand how miRs are identified to interpret the literature. As indicated, "mir" precedes the name of a pre-miR while "miR" precedes the designation of a mature miR. Over the years, specific miRs have been numbered to distinguish and identify them. Usually, the numbers range from 1 to 9999; the small numbers designate miRs that were identified earlier. An initial three letter prefix may refer to species associated with the miR. Some species designations are listed in Table 2. Thus the designation for miR 130 in humans could be "hsa-miR-130," while the miR 130 in mice would be designated "mmu-miR-130." A pre-miR may produce miRs from different ends of the molecule; if one of two miRs comes from the 3′ end, it is designated, -3p, and if from the 5′ end, -5p.

When mRs differ by only one or two nucleotides from the form of miR identified originally, the related miRs are designated by a letter, e.g., "a," "b," "e," and in some cases where there are three very similar miRs as "b#." Also, if two miRs come from the same pre-miR, but one is the minor component, it may have been labeled previously as "*." Thus, miR-130* is the minor component and miR-130 is the major form of miR-130 in a cell; however, designations recently have been changed to -3p or -5p to describe such forms. Examples of various designations of miRs are shown in Fig. 1.

Frequently the species designation, hsa, is omitted for miRs for studies of human specimens and species prefixes often are deleted if a publication is limited to one species. Of great use to investigators studying miR is the web site, "mirbase.org," currently 2011 version 18, that can be used to search for specific miRs or information about miRs. This web site contains more than 18,000 entries concerning more than 150 species and is organized to be accessed using several approaches. One approach is based on "species: chromosome: sequences." If one enters *Equus caballus* (horse), for example, and chromosome 2, 18 "mirs" are listed, and each represents the pre-form of the miR. Specifically, one finds eca-mir-30e on chromosome 2. The mirbase.org site was less useful, however, for searching for miRs involved in cancer. By contrast, the "Human MicroRNA Disease Database" and the "miR2 disease database" are much more useful concerning the literature related to the involvement of miRs in diseases in general and neoplasia specifically (Jiang et al. 2008, Lu et al. 2008). These databases can be searched by organ, cancer or type of cancer, e.g., carcinoma, but sometimes must be searched under "neoplasia."

Table 2. Species and designation for miR

Species	Designation
human	hsa
mouse	mmu
rat	rno
sheep	oar
dog	cfa
chicken	gga
viral	__v
Drosophilia	d__

Frequently, miRs provide a mechanism by which the amounts of proteins can be down-regulated. This function of miRs may occur by facilitating the degradation of mRNAs, which inhibits the translation of mRNAs or the transcription of mRNAs. Sometimes the mRNAs and their associated proteins that are down-regulated are involved primarily in the metabolism or degradation of important driver genes. For example, some proteins are controlled primarily by the degradation of the protein product (e.g., $p27^{kip\text{-}1}$ metabolized by Skp-2); thus, if miRs inhibit the metabolic enzyme targeting a phenotypically important protein, the phenotypically important molecule would be expected to increase. Similarly, miRs can be inhibited by methylation of their promoters as well as by proteins from other genes, and less commonly, may stimulate translation directly and thus increase specific proteins (Vasudevan et al. 2007).

Because miRs function in the normal development and growth of cells, they would be expected to be dysregulated in disease processes and are likely to be important for at least some aspects of all human diseases. We are just beginning to understand their importance in human diseases (Jiang et al. 2008, Lu et al. 2008). As expected, because miRs frequently are involved in developmental processes, congenital malformations of many organ systems, including the heart and brain, may occur if miRs are dysregulated.

Abnormalities related to miRs may occur at several levels including deregulation of the pri-miR, pre-miR, or mature miR. Other types of dysregulation may occur by modifications of the target 3′-UTR of mRNAs that affect the binding of miRs. Specific changes in the mRNA may cause the 3′-UTR no longer to bind a specific miR or it may cause binding to the 3′-UTR of a previously unbound miR. All the possibilities above may occur as somatic mutations or gene dysregulation during the development and progression of specific cancers or as germ line mutations by which such abnormalities are transmitted to offspring and result in familial diseases.

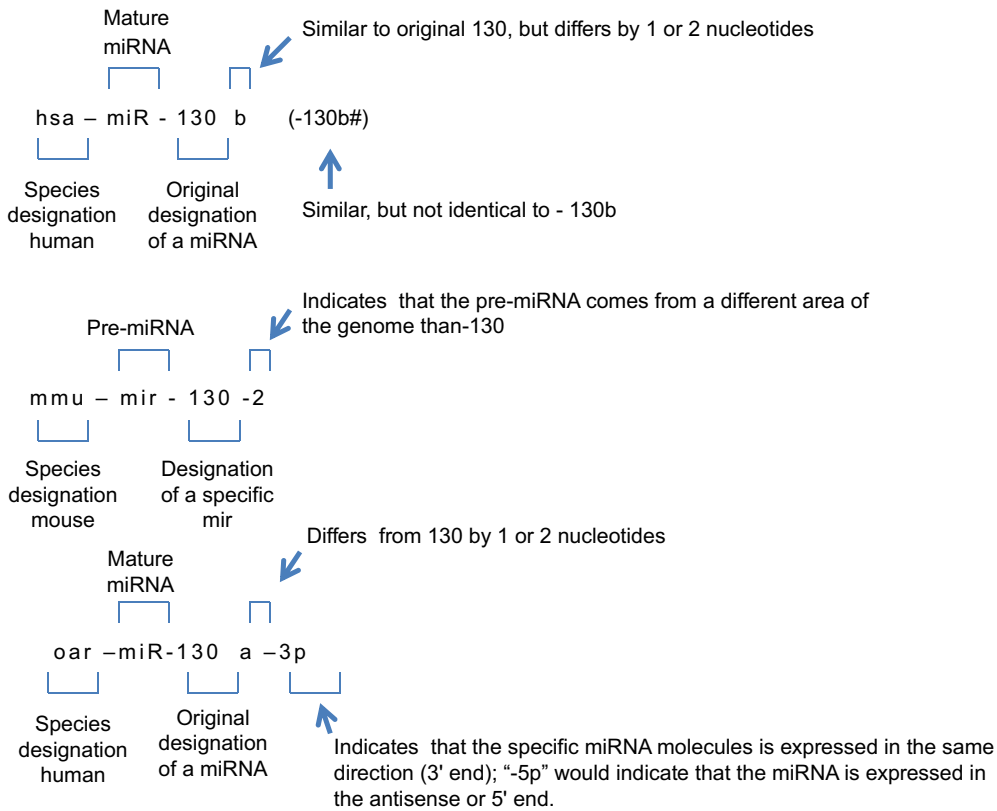

Fig. 1. XXX.

MiRs and exosomes

MiRs represent one of several newly described categories of molecules and pathways that "fine tune" cellular functions. MiRs can be transcribed and function not only within cells, but they also can be transferred to cells as a form of intercellular communication. This can be accomplished by packaging of miRs in membrane bound vesicles that are released from cells into the interstitial space. The vesicles within interstitial spaces may act locally through autocrine, paracrine or other-crine activities, or they may be picked up by blood or other bodily fluids to provide endocrine-like signals to distant cells (Kosaka and Ochiya 2012). The exosome is one type of vesicle that has been reported to contain molecules of miR. Exosomes are bilayered-membrane-bound nanovesicles that are released from the vesicular bodies of normal and diseased cells. Exosomes are present in most body fluid including blood. In blood, exosomes typically are 30–100 nm in diameter, have been described as "cup-shaped" and they express specific molecules such as the tetraspanins, CD9, CD63 and CD81. Although the details are uncertain, the molecular features of the external surfaces of exosomes control their uptake by cells, hence the effects of their contents on cellular functions (Zhang and Grizzle 2011).

Exosomes have been reported to contain functional proteins, mRNAs and lipids as well as miRs. Again, the details about how these molecules are sorted selectively into exosomes remains elusive. Nevertheless, typical exosomes contain hundreds of miRs and the packaging of miRs in exosomes may protect them from RNases (Kosaka and Ochiya 2012). How specific miRs in exosomes function and provide signals to cells is uncertain.

The molecules contained in exosomes, especially the proteins, peptides and mRNA, have been reported to be characteristic of the cells from which they arise. Because tumors secrete exosomes, i.e., tumor-derived (TD) exosomes, their contents, especially proteins, peptides, mRNA and miRs, have been found to be characteristic of the tumors from which they arise. Because TD exosomes contain miRs, it has been proposed that these miRs can be used as biomarkers that are useful for translational research focused on diagnosis, risk assessment, prediction, measurement of therapeutic responses and determination of prognosis (Lu et al. 2005, Grizzle et al. 2012, Zhang and Grizzle, in press). Circulating TD-exosomes of patients with ovarian cancer

have been reported to contain eight miRs whose expressions were significantly distinct from those observed in benign diseases (Taylor and Gercel-Taylor 2008). In many cases, the miRs were expressed differentially in exosomes compared to the matching fluids; thus, miRs contained within exosomes may provide greater sensitivity and specificity for translational studies (Kosaka and Ochiya 2012, Nair et al. 2012).

Other small RNAs

SiRNAs are double-stranded small RNAs that are present in prokaryotes, plants and animals evolutionarily below worms where they protect against certain viruses and intracellular parasites. In some cases, this double-stranded RNA attracts a protein complex containing dicer, which cleaves the double-stranded RNA. Like miRs, they are bound by Argonaute and other molecules into an RNA-induced silencing complex (RISC), which then is processed as described previously for miR and whose action may be similar to miRs. Alternatively, siRNAs may be bound with Argonaute into a different complex, the RNA induced transcriptional silencing complex (RITSC), which can inhibit transcription of genes.

There are other endogenous forms of small RNAs that also act post-transcriptionally on mRNA and are related to, but currently are considered distinct from, miRs (Lee et al. 2010, Naqvi et al. 2009). Small RNAs that mimic siRNA may be synthesized exogenously and used experimentally to decrease specific mRNAs, hence, proteins; these also typically are called siRNAs (Devi 2006).

MicroRNAs in cancer

Pre-invasive and invasive neoplastic cells typically hijack embryological and repair processes and pathways to facilitate neoplastic development and progression. Pathways controlling proliferation, apoptosis, cellular motility, cellular invasiveness and cellular orientation (polarity) are typical pathways that are dysregulated in neoplastic cells to facilitate their growth and survival. While pathways controlling proliferation and apoptosis, for example, are regulated carefully in normal tissues, these pathways frequently are dysregulated in neoplasia. Also, with increased proliferation and dysregulated apoptosis, mutations in genes, overexpression of genes, and changes in the epigenetic control of transcription may develop in neoplasia.

MiRs are involved in most aspects of neoplasia from the development of neoplastic lesions to the spread of cancer by metastasis. MiRs involved in cancer have been designated as "oncomiRs" (Cho et al. 2007, Esquila-Kerscher and Slack 2007, Lujambio 2009). OncomiRs can modulate the development, progression and dissemination of neoplastic processes by acting as either tumor suppressors (e.g., miR-34a) or oncogenes (e.g., mir-17-92 cluster). It is of interest that the tumor suppressors, miR-18a, miR-34b/c and miR-9, can be silenced by hypermethylation and this silencing facilitates nodal metastasis (Cho et al. 2007, Lujambio 2009).

MetastamiRs are miRs that are involved specifically in the metastatic process (Hurst et al. 2009, White et al. 2011, Lopez-Camarillo et al. 2012). They frequently function as an intermediate signal in pathways that inhibit or facilitate cancer, e.g., miR146a/b acts downstream of the BRMS1 gene, which suppresses metastasis in breast cancer, but miR146a/b acts prior to genes that are identified as regulated by BRMS1 (Hurst et al. 2009). Other miRs involved in metastasis of breast cancers include miR-335, miR-126, miR-10b, miR-9 and miR-155 (Tavazoie et al. 2007, Ma et al. 2007, 2010, Negrin and Calin 2008, Xiang et al. 2011). Similarly, miR-31 usually acts as a general tumor suppressor as well as a suppressor of metastasis by its action on integrin-α5, radixin and RhoA, while the miR-200 family (miR -200a, b, and c, miR-141 and miR-429) may inhibit or facilitate metastasis depending on whether effects of epithelial to mesenchymal transition or mesenchymal to epithelial transition dominate (Dykxhoom 2010).

MiRs have been claimed to be useful for early detection, diagnosis and prognosis of various cancers and for management of patients by prediction of therapeutic efficacy, monitoring responses to therapy or as targets for therapy (Mak et al. 2005, Waldman and Terzic 2007, Martello et al. 2010, Manne et al. 2010, Shah et al. 2009). It has been suggested that MiRs potentially are important clinically for most cancers and specifically for cancers of the ovary (Shah et al. 2009, Taylor and Gercel-Taylor 2008), lung (Rabinowitz et al. 2009, Mallick et al. 2010), breast (Pigati et al. 2010, Xiang X et al. 2011), pancreas (Wang et al. 2009, Srivastava et al. 2011), prostate (Coppola et al. 2010) and brain (Delfino et al. 2011).

Small non-translatable RNAs such as miRs now are recognized as an important group of regulatory molecules that are involved primarily in post-transcriptional regulation of genetic information.

There are thousands of different miRs that bind to the untranslated 3′ ends of mRNAs and thereby modulate the degradation of these mRNAs and inhibit their translation. MiRs can be carried within exosomes to provide autocrine, paracrine, and endocrine type intercellular signals among normal and diseased cells, and especially neoplastic cells. MiRs also are involved in disease by their dysregulation. MiRs likely represent one of the biological pathways that neoplastic lesions use to promote their development, progression and dissemination; therefore, these molecules may be attractive targets for novel approaches to therapy, diagnosis and prevention of cancer.

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Biomarkers and the genetics of early neoplastic lesions

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Abstract. It has become increasingly evident that the study of DNA is inadequate to explain many, if not most, aspects of the development and progression of neoplastic lesions from pre-invasive lesions to metastasis. Thus, the term "genetic" can no longer refer to just the study of the genome. Much of the action in genetic research now shifts to the methods by which the pre-mRNA from one gene is processed to yield multiple different proteins, different quantities of the same protein as well as other forms of regulating RNA. Thus, the age of post-transcriptional processing and epigenetic control of the transfer of information from the genome has arrived. The mechanisms of post-transcriptional processing and epigenetic control that must be characterized in greater detail including alternate splicing, regulation of mRNA degradation, RNA regulatory factors including those factors which extensively edit mRNAs, control of translation, and control of protein stability and degradation. This chapter reviews many of the processes that control information from the genome to proteins and how these factors lead from less than 40,000 genes to more than an order of magnitude increase more proteins which actually control the phenotypes of cells – normal or neoplastic. It is usually the products of genes (e.g., mRNA, microRNA and proteins) that are the molecular markers that will control translational research and ultimately, individualized (personal) medical approaches to disease. This chapter emphasizes how the process of neoplasia "hijacks" the normal processes of cellular operations, especially those processes that are important in the normal development of the organisms – including proliferation, cellular death, angiogenesis, cellular mobility and invasion, and immunoregulation to ensure neoplastic development, survival and progression. This chapter reviews the wide range of processes controlling the information that flows from the genome to proteins and emphasizes how molecular steps in pure processes can be used as biomarkers to study prevention, treatment and/or management of diseases.

Keywords: Intraepithelial neoplasia, dysplasia, methylation, microsatellite instability, mutations, insertions, deletions, oncogenes, suppressor genes, aneuploidy, translocations, caretakers, gatekeepers, landscapers, mismatch repair genes, LOCDIR, epigenetics, immunoregulation, tumor associated fibroblasts, exosomes, angiogenesis, clonal selection, viral insertions, biomarkers, validation

1. Introduction

Two great challenges in cancer diagnosis and prevention are 1) the detection of specific pre-invasive neoplastic lesions that give origin to malignant tumors and 2) the identification of those prognostic factors of tumors that predict the outcome of individual cancer patients. As personalized medicine becomes more established, the use of biomarkers to predict responses to various therapeutic regimens also will become much more important. Also, the ability to detect neoplastic transformation at its earliest presence is a requisite for improving preventive interventions and reducing cancer incidence.

In this chapter we will discuss the molecular aspects of early pre-invasive neoplastic lesions; specifically, alterations in genes regulating proliferation, differentiation, apoptosis and invasiveness as well as chromosomal aberrations and microsatellite instability will be addressed. We will argue that the identification of molecular changes associated with neoplastic transformation will lead to the development of new molecular markers for the early detection of pre-invasive neoplastic and malignant lesions, assessment of cancer risk, assessment of responses to preventive or therapeutic inter-

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ventions, and separation of less aggressive neoplastic lesions from more aggressive lesions.

1.1. Early neoplastic lesions: Definition and general concepts

Neoplastic lesions may derive from epithelial or nonepithelial cellular populations. Often, pre-invasive neoplastic lesions are small, multiple and may display wide ranges of diversity [1]. Morphological alterations characterizing pre-invasive neoplastic lesions include nuclear pleomorphism consisting of increased variation in nuclear size, shape, and hyperchromatism, abnormal mitoses, abnormal nucleolar patterns and altered or absent differentiation. The evolution of these types of lesions may take three directions: spontaneous regression, progression toward a fully malignant phenotype, or stasis, remaining as a pre-invasive neoplastic lesion [2]. Although there are discrepancies in terminology used by pathologists to describe these types of early neoplastic lesions, they are most frequently referred to as pre-invasive neoplasia, dysplasia, carcinoma *in situ*, intraepithelial neoplasia, or as incipient cancer [3,4].

Based exclusively on morphological features, lesions which progress to malignant tumors cannot be easily distinguished from lesions which spontaneously regress. It has been shown that normal epithelium and normal-looking tissue in the proximity of a tumor may carry mutated cancer genes, suggesting that the first steps in early pre-invasive neoplasia do not have a distinctive morphologic correlate [5–8]. Similarly, molecular changes in lesions known to be at risk for developing morphologic pre-invasive neoplasia such as ulcerative colitis, actinic skin damage, and Barrett's esophagus, may precede histomorphologic changes by many months [9,10]. Development of molecular markers, which clearly reflect various stages of neoplastic transformation, may redefine the diagnostic criteria for precursor lesions of cancer [11].

Carcinogenesis is an indivisible continuum of molecular and morphological changes that may culminate in the development of invasive tumors. A growing body of evidence suggests that a tumor may initially start as a stem cell that develops mutations and subsequently produces a clonal population of cells harboring these and additional mutations which uniquely suit the clone to its microenvironment. Because early neoplastic lesions exhibit phenotypic heterogeneity, multicentricity, genetic instability and other chromosomal alterations, it is not always easy to predict their biological behavior or risk of progression [12]. Studies in which the molecular features of pre-invasive neoplastic lesions are correlated with molecular features of matching invasive lesions, for example, a colorectal cancer with an adenomatous polyp forming its edge, should be useful in identifying molecular features associated with greater risks of progression [13]. Similarly, studies of prognostic factors in specific cancers may identify molecular features associated with aggressive behavior [14]. Good transgenic mouse models of a cancer (e.g., pancreatic cancer) also may be useful in identifying molecular features leading to the development of metastatic disease [15,16]. In some cases it is the combination of molecular changes, not one molecular change, that leads to aggressive behavior. For example, in mouse models, mutations in K-ras frequently lead only to pancreatic intraepithelial neoplasia (PanIN) and only loss of p16/p19 (Ink4a/Arf) may not cause major changes in transgenic mice; however, activation of K-ras-2 by mutation plus loss of Ink4a/Arf leads to metastatic pancreatic cancer [16]. Of note, some molecular features in one tumor may identify an aggressive subset of tumors, but these same markers e.g. p53, may not identify aggressive features in a different tumor [13,17]. The study of early neoplastic lesions is further complicated by observations that cells carrying chromosomal and genetic alterations may disappear during the spontaneous regression of intraepithelial neoplasia, as reported for cervical, skin and bronchial lesions [1,2, 17].

Thus it is apparent that molecular changes precede histological and morphological alterations and these findings may have implications for developing a new classification of early neoplastic lesions, based on molecular parameters [11]; however, unless there are grossly observable histopathologic changes (e.g., increased vascularity or inflammation), that mark areas in which molecular changes are likely, molecular changes alone are unlikely to be of practical use in identifying the boundaries of pre-invasive neoplastic lesions because the pattern of sampling would be too complex and the many assays to demark the boundary of the lesion would be too costly (Figs 1 and 2).

1.2. Molecular basis of early neoplastic lesions

Because a proportion of high grade pre-invasive neoplastic lesions progress to frank cancers, pre-invasive neoplastic lesions must develop part, but not all of the features of the associated cancers. The six "hallmarks of cancer" as proposed by Hanaken and Weinberg [18]

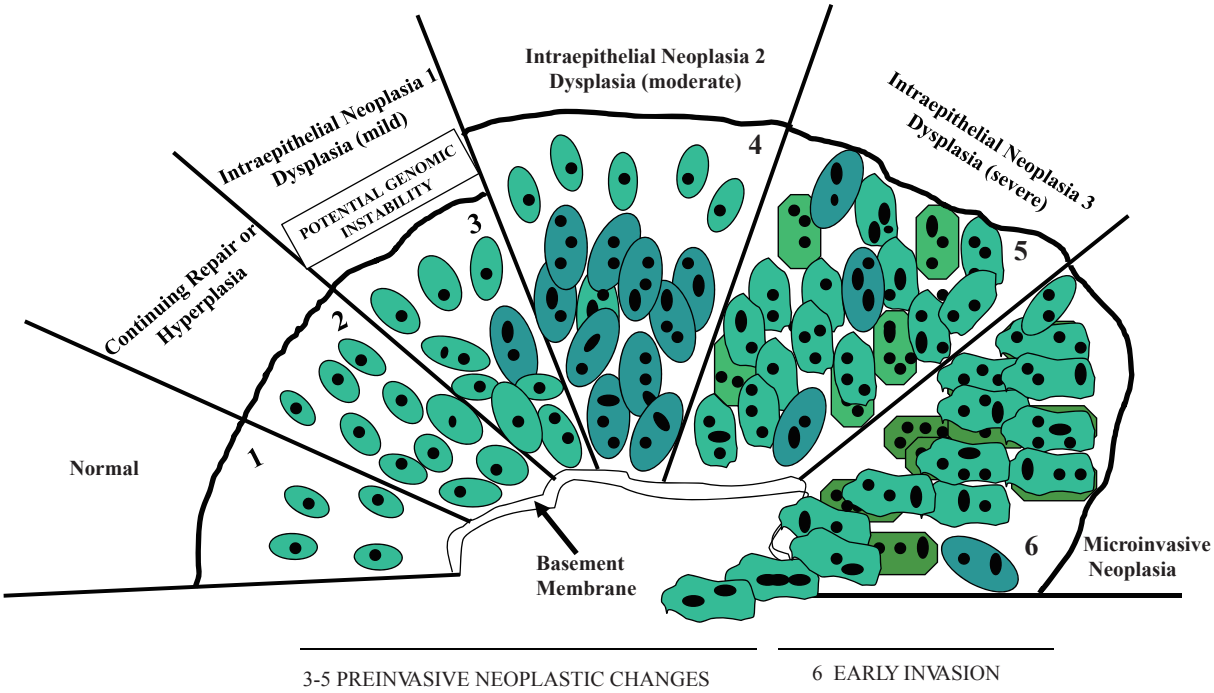

Fig. 1. This figure demonstrates the histologic features of epithelium that represent changes that can lead to invasive cancer. Usually hyperplasia develops which may be caused by **l**ongstanding **c**ontinuing **d**amage **i**nflammation and **r**epair (LOCDIR) or by unidentified stimuli. In this process, molecular changes may develop, including factors affecting mechanisms of genetic repair leading to various degrees of intraepithelial neoplasias (dysplasia) and ultimately to locally invasive cancer. This cartoon demonstrates development in cellular crowding and disorganization as is indicated by cellular nuclei. In the more severe forms of intraepithelial neoplasia, the whole thickness of the epithelium may be involved. The black areas demonstrate nucleoli and other chromatin features within the nuclei which become more prominent and variable in neoplastic processes. Cell boundaries are not demonstrated.

also must apply in part to pre-invasive neoplasia. Actually, an additional hallmark of cancer not listed by Hanaken and Weing berg should be the ability of neoplastic lesions to evade partially or completely immune surveillance. Thus, the most important hallmarks of pre-invasive lesions are:

1) Self sufficiency in growth signals
2) Evasion of apoptosis
3) Insensitivity to anti-growth signals
4) Evasion of immune surveillance
5) Limitless replicative potential

Because pre-invasive neoplastic lesions are in situ and some of these lesions regress, the following hallmarks of cancer are less likely to be important to the maintenance of pre-invasive neoplasia.

6) Sustained angiogenesis
7) Capability for tissue invasion and metastasis

Molecular alterations involved in human carcinogenesis are very diverse, as are the mechanisms by which cellular functions may be altered [4,19,20] (Table 2). It has been implied that an increased rate of mutation could be the most important factor in tumorigenesis although this assumption is not universally accepted [21–24]. In any case, mutations in genes controlling DNA repair and DNA synthesis appear to compromise the genetic stability of cells and contribute to the progression of neoplastic changes, which, in turn, leads to the increase of genetic alterations in emerging clones. Mutations that provide a selective growth advantage appear to play a key role for carcinogenesis [25]. Based on the key factors that lead to cancer, early neoplastic transformation would rely on molecular changes that avoid apoptosis and facilitate continued proliferation by not responding to factors that inhibit growth and developing combinations of factors that self-sufficiently stimulate growth that is not limited in its replicative potential. Unlimited growth is supported via the maintenance of the lengths of telomeres by the action of telomerase which is controlled by multiple trans-activating transcriptional regulators such as myc [18,21]. For example, a setting of long standing continuing damage, inflammation and repair (LOCDIR) which occurs in

Table 1
Precancerous Lesions and Conditions

Lesion type	Examples
Non-Neoplastic Precancerous Lesions	Endometrial hyperplasia
	Barrett's esophagus
	Intestinal metaplasia
	Atypical hyperplastic lesion in breast
	Squamous metaplasia (Bronchus)
	Cirrhosis of liver
Precancerous Conditions	Inflammatory bowel disease
	(e.g., ulcerative colitis)
	Chronic gastritis
	Chronic pancreatitis
	Chronic cholecystitis
	Leukoplakia of oral cavity
Pre-invasive Neoplastic Lesions	Prostatic intraepithelial neoplasia (PIN)
(Dysplasia, Intra-epithelial Neoplasia)	Esophagus (squamous or columnar epithelial dysplasia)
	Gastric (adenomas)
	Colonic (adenomas and flat adenomas)
	Endometrial intraepithelial neoplasia (EIN)
	Cervical intraepithelial neoplasia (CIN)
	Ductal or lobular carcinoma in situ
	(DCIS/LCIS)
	Pancreatic intraepithelial neoplasia (PanIN)
	Oral cavity (squamous dysplasia)
	Larynx (squamous dysplasia)
	Lung (bronchoalveolar in situ carcinoma,
	atypical adenomatous hyperplasia,
	squamous dysplasia)

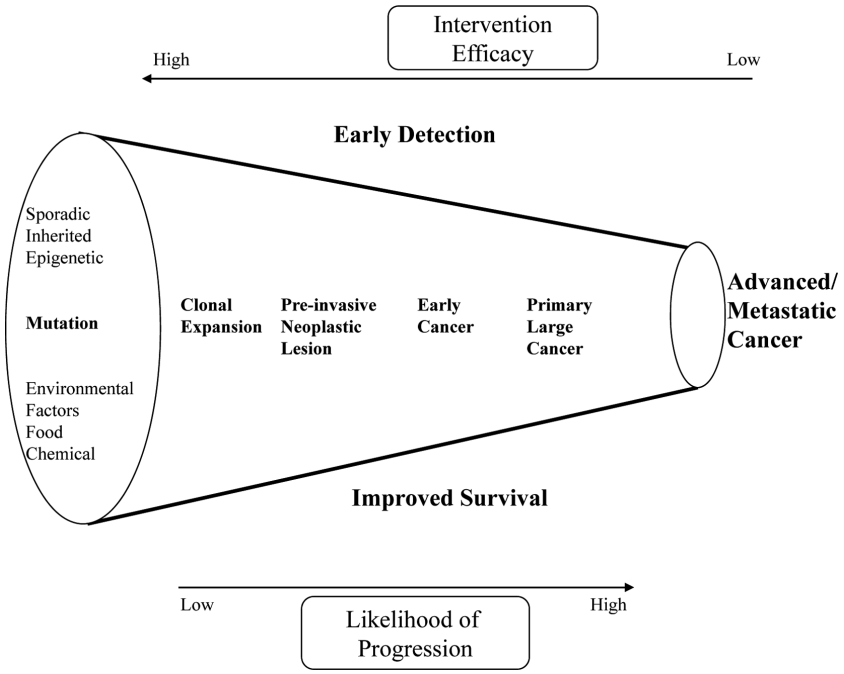

Fig. 2. Early Detection Tunnel: The impacts on efficacy of intervention and survival of early detection of neoplastic lesions.

conditions such as ulcerative colitis, puts pressure on stem cells to proliferate [4]. This proliferation occurs in a setting in which factors are present (e.g., reactive oxygen and reactive nitrogen species) that inhibit complete repair which may lead to genetic damage to stem cells. When such genetic changes develop in a specific

Table 2
Molecular alterations commonly found in human cancer

Type of mutation	Possible causes of mutation	Possible consequences of mutation
Single Base Change	Carcinogens	Missense: may alter gene function
	Defective repair genes	Nonsense: truncates gene
Methylation	Defective methylation gene	Gene silencing or inappropriate expression
Small insertions or deletions	Carcinogens	Frameshift: truncates gene
	Defective DNA repair genes	
Microsatellite instability	Defective DNA repair genes	Frameshift when inside gene
		Alters mRNA stability; differentially effects specific genes,
	e.g., TGFβRII	
	Defective DNA repair genes	Loss of gene function of multiple genes
	Defective DNA replication genes	
Large deletions	Illegitimate recombination	
	Double strand break, with incorrect rejoining	
	Homologous recombination	
	Defective DNA repair genes	Overexpression of gene
DNA amplification	Defective DNA replication genes	
	Illegitimate recombination	

stem cell and these changes favor rapid and continuing growth of the specific stem cells in their microenvironment, a pattern of clonal growth may develop. When this clonal growth also develops methods to produce sustained angiogenesis, invasion, and metastasis, a malignant tumor may develop. The clonal progeny of a stem cell may subsequently develop alterations in gene expression that lead to the development of sustained growth [12,18,21,26]. It has been postulated that clonal selection continues throughout various stages of tumor growth, which may explain the sequential emergence of altered populations of cells over time [25]. For example, such a hypothesis of clonal selection is in agreement with the emergence of multiple pre-invasive neoplastic lesions, and sometimes second primary tumors. This pattern is known as "field effects" that can be induced by carcinogen-exposure, as first described by Slaughter [27]. This is discussed further in a subsequent section.

Epigenetic changes, in particular the methylation of DNA, as well as factors in the tumor microenvironment, such as hormones, vitamins, prostaglandins, growth factors and cytokines, play an important role in neoplastic transformation. These factors may markedly influence the evolution of pre-invasive neoplastic cells by accelerating, retarding, or inhibiting their transformation into fully malignant cells, or even reversing their characteristics to a normal phenotype [28]. For example, it has been shown that in an organ such as the prostate that circulating cytokines released by underlying stromal cells may modulate normal epithelial differentiation, proliferation, ductal morphogenesis and protein secretion [29]. In turn, secreted factors from prostatic epithelial cells may alter the underlying stroma, which supports the hypothesis that dysregulation of the cross-talk between epithelial and mesenchymal circulating cytokines is involved in carcinogenesis [30].

Another major feature of all malignant tumors is the partial avoidance of the effects of death of the neoplastic cells. Tumor growth is caused by the unequal balance between cellular proliferation and cellular death. In cancers, there may be several types of cellular death, e.g., ischemic necrosis; however, most forms of individual cellular death are sometimes incorrectly combined under the term apoptosis – a death of the cell directed by endogenous causes which affect the cellular DNA. Thus, one of the features required for neoplastic lesions to progress to frankly malignant tumors is described as avoidance of apoptosis [18]. Apoptosis is a cellular death induced by an intracellular program attacking DNA and nuclear and cytoplasmic proteins while leaving cellular membranes intact. There are two main pathways leading to apoptosis – one via the extrinsic pathway (death receptor initiated) and one via the intrinsic pathway (mitochondrial associated pathway). There are multiple stimulatory and inhibitory factors that affect either or in some cases, both of these pathways. For example, there are more than 20 proteins in the Bcl-2 family which regulate apoptosis primarily via mitochondrial associated pathways. The two main anti-apoptotic proteins are Bcl-2 and Bcl-X and the three main pro-apoptotic proteins are Bak, Bax and Bim. Thus, apoptosis tends to be a balance of the various stimulatory and inhibitory proteins associated with the pathways of apoptosis. Nevertheless, one has to be very careful in interpreting the effects on differential expression of these molecules on tumors. For example, increased levels of Bcl-2 are associated with

a good prognosis in colorectal and breast adenocarcinomas, but with a bad prognosis in prostate, bladder, and hematopoietic malignancies [31–33]. Similarly, the p53 mutation has been associated with normal appearing skin [10] and with pre-invasive neoplastic lesions of tissues such as breast, skin and SCC of the oral cavity, while in other types of tissue mutations of or dysregulation of p53 has been associated with advanced or metastatic lesions, e.g., prostate [34].

Recent findings suggest that apoptosis is defective in neoplastic cells and that the failure of cells to die in response to damage may permit the progression of the pre-invasive neoplastic lesion to frank malignancy during the pre-invasive state. Impairment of apoptosis is involved at very earliest stage of neoplasia, as demonstrated in colonic epithelial cells harboring an APC mutant gene [35].

The study of oncogenes and tumor suppressor genes has provided critically important information for our understanding of the molecular events underlying the sequence of changes leading to tumor development [36]. Mutations, which may induce the activation of oncogenes or inactivation of tumor suppressor genes, may dysregulate cellular proliferation through a variety of mechanisms. Oncogenes and tumor suppressor genes may affect differentiation, apoptosis, signal transduction, intercellular communication and adhesion, initiation of DNA replication and regulation of expression of certain specific genes [36,37]. Alterations in other classes of genes, including those regulating the interaction with the extracellular matrix, as well as neoangiogenesis, may enable cells to acquire an invasive and metastatic capacity [38–42]. It is clear that molecular manifestations of neoplastic transformation precede detectable morphological changes and that genomic changes associated with cancer may occur in cells long before morphological alterations become apparent.

Table 3
Biomarkers involved in regulation of proliferation, differentiation, apoptosis, senescence and DNA repair detected in premalignant neoplastic lesions

Examples of biomarkers in premalignant lesions
Proliferation
Ki67
PCNA (Proliferating Cell Nuclear Antigen)
Creatine kinase
Cyclin E
Cyclin D
Bcl-1
Mitotic rate
Oncogenes
$p185^{erbB2}$
cKi-ras
c-myc
erbB1(mutated EGFr)
H- or K-ras mutations
Tumor suppressor genes
p53
Rb
p16 (Inka4)
p19 (Arf)
Growth factors
TGF-alpha
EGFR
TGFβ
TGFβR1
TGFβR2
IGF1R
IGF2R
ERP28
Differentiation
CD44v6
CDw75ag
Apoptosis
Bcl-2
Bax
Senescence
Telomerase
DNA Repair
MSH3
MSH6
PMS1

2. Biomarkers of early neoplastic lesions

2.1. General concepts

Biomarkers include any morphological, biochemical or genetic alteration by which a physiological or pathological process can be recognized and monitored. Cancer related biomarkers include those markers which are associated with early neoplastic lesions, as well as the markers associated invasive or metastatic tumors (Table 3). Presumably, molecular events in the causal pathway leading to cancer will be identified by a relatively restricted set of markers. A panel of markers reflecting invasion-related molecular changes will aid in characterizing advanced or metastatic cancers. In the following section, we summarize some critical events that occur during the initiation of pre-invasive neoplasia.

In general, the direction of the differentiated expression (increased or decreased) of biomarkers in pre-invasive neoplastic lesions follow that of invasive lesions. Thus, if p53 is mutated in a colorectal polyp from which a colorectal cancer (CRC) arises, the CRC will usually contain the same mutation in p53 [13]. Similarly, if there is strong membrane expression of

$p185^{erbB-2}$ in ductal carcinoma in situ (DCIS) of the breast, there is usually similar expression in the associated ductal carcinoma. Also, in general, if biomarkers are high in the majority of pre-invasive neoplastic lesions, they will be high in the metastatic lesions of the same type of tumor; however, there are some exceptions such as the expression of TAG72 (B72.3) in prostate cancer [43].

2.2. Chromosomal aberrations

Common chromosomal aberrations which may be observed in metaphase preparations after treatment of cells with DNA-damaging agents include breaks, deletions, translocations, amplifications, duplications, circularizations and dicentrics. Some chromosomal aberrations, such as translocations or deletions tend to be stably transmitted throughout generations of cells. In contrast, other chromosomal aberrations, such as dicentrics, are not passed on the next cell generation, which suggests that they represent an unstable event, caused by a recent clastogen exposure. Studies of changes in chromosomes have added in many ways to our understanding of tumor development and have increased the evidence for the importance of cumulative genetic alterations as a major force in tumor evolution [44,45].

2.2.1. Aneuploidy

Aneuploidy is the most common chromosomal aberration associated with premalignant lesions [46–48]. Aneuploidy, or the change in copy number of the chromosomes, is often measured as the DNA index, a ratio of the DNA content of a cell to that of a diploid normal cell. A normal DNA index can be seen in cells having considerable gains or losses in various different chromosomes, and indeed allelic loss at a particular site is often accompanied by no cytogenetically detectable changes in the remaining chromosomes. Based on DNA content, a tumor can be diploid but still may have changes in gene copy numbers or allelic imbalances. Cells that have aneuploidy in DNA may have chromosomal numbers below diploid or above diploid. When the number of chromosomes is twice the diploid number, the cell is described as tetraploid. Octoploid is 4 times diploid. Tetraploid and octoploid cells can occur in normal liver, especially in rodents. Aneuploidy as well as point mutations appear to be associated with conditions predisposing to cancer. Aneuploidy may have value as a complement to histological examination in the surveillance of ulcerative colitis patients, who are known to have an increased risk of developing colorectal cancer [49].

2.2.2. Deletions and translocations

A deletion usually refers to a large loss of a chromosomal region or even of an entire chromosome; it thus may involve the loss of a single copy of each of many contiguous genes. When such a loss occurs, there can also be an associated reduplication of the remaining chromosomal copy of the lost area or uniparental disomy [50,51]. Such acquired uniparental disomy (UPD) usually results in the under estimation of LOH. In UPD, there is initially LOH, thus a section of a chromosome from one parent is lost leaving the same area on the chromosome from the other parent intact. By methods which are not understood, one or more new copies of the area of chromosomal deletion are reproduced from the remaining area of the chromosome that matches the lost chromosomal area. Thus, there will be at least two copies of the originally deleted chromosomal area, but both copies will be from one parent, i.e., the parent whose chromosomal area was not deleted.

Clinically UPD may be important in that genes may be amplified secondary to UPD and polymorphic proteins may be homozygous more than would be expected. Such changes will not easily be detected [50]. Of interest, BRCA1/2 associated serous ovarian carcinomas exhibited more genetic instability and more UPD than randomly selected sporadic serous ovarian carcinomas [51] as would be expected because of the genetic instability induced by BRCA1/2.

Alternately, loss of one parental copy of an area of a chromosome can be accomplished by mitotic recombination with the homologous chromosome, again resulting in a retention of cytogenetically normal structure of the chromosome. Therefore cytogenetic analysis can underestimate the extent of genetic losses. It is consequently more instructive to speak of the loss of one parental copy of a gene, of heterozygosity (LOH) or allelic loss. Deletion can also be the mechanism for loss of both copies of a gene (homozygous deletion). This usually combines a large LOH deletion with a much smaller deletion that involves one gene or scores of genes. For example, when one copy of p53 is lost, there is frequently a loss of the other copy, resulting in a homozygous deletion of p53. Because the homozygous deletions of a neoplasm obviously cannot involve any of the genes that are essential for cellular metabolism, replication or survival, their size and prevalence are highly restricted by the types of neighboring genes as well as whether or not there is UPD.

When a LOH involves a major suppressor gene such as p53 or Rb and no other genes critical for cell survival are lost, this genetic change usually results in an

increase in cellular functions and/or loss of apoptotic responses. Such changes can lead to neoplasia. Recently, it has been recognized by the Czerniak laboratory [52,53] that in some cases major suppressor genes are surrounded geographically on the chromosome by less critical suppressor genes. A loss of one or more of these minor suppressor genes may occur as an early change of neoplasia; it may stimulate a clonal expansion and may lead to a loss of the major suppressor gene (e.g., Rb). These genes have been designated as "forerunner" genes [52,53]. Loss of forerunner genes in the earlier stages of neoplasia followed by the loss of the major associated suppressor gene appears to be common as early neoplastic lesions progress [52,53].

Translocation involves a rearrangement of a portion of one chromosome to another chromosome or a rearrangement within a single chromosome. As a result, the activation of an oncogene may occur. For example, a growth-promoting gene can be placed near a strong or constitutively active transcriptional promoter. Alternately, the introns of two different genes could be united so that the spliced transcripts of the rearranged site produce a fusion of peptide domains, encoding a novel protein [54,55]. Perhaps the best known translocation is represented by the Philadelphia chromosome which is present in chronic myelocytic leukemia. It results from translocation of the ABL-1 proto-oncogene positioned on chromosome 9, to a region of chromosome 22 downstream on BCR. This fusion activates the ABL-1 gene resulting in either chronic myeloid leukemia or acute lymphoblastic leukemia, depending upon the chromosomal breakpoint [56]. A vast number of chromosomal alterations associated with hematopoietic cancer, sarcomas and premalignant lesions have been reviewed elsewhere [56,57]. Such fusion genes were thought not to occur in sporadic carcinomas until Chinnaiyan reported the presence of the TMPRSS2-ERG fusion gene in carcinoma of the prostate [58,59]. Other similar fusion genes, e.g., TMPRSS2-ETVI, also have been identified in the prostate. Although controversial, some groups have reported that TMPRSS-ERG is an important prognostic factor for radical surgery of the prostate [60]. Since the discovery of fusion protein in prostate carcinoma, several other fusion genes have been reported in lung and other carcinomas [61].

2.3. *Actions of suppressor genes – Caretakers, gatekeepers and landscapers*

Complex animals and humans successfully prevent the development of cancers via multiple mechanisms at the cellular, tissue (micro-environmental) and organismal levels. It may take several decades to arrive, via clonal selection, at even a pre-invasive neoplastic lesion and even these relatively advanced neoplastic lesions may regress by mechanisms which are largely unknown. An excellent example is the mutations of p53 that are present in sun exposed skin. Skin carrying these mutations may represent up to 4% of the skin of a human [10,62] yet the pre-invasive neoplastic lesions which result from mutations in p53, actinic keratoses (AK), only develops in a small percentage of skin and even most AK lesions do not develop into invasive cancers; also, clones with p53 mutations as well as AK lesions may regress [62–64].

The genetics of early neoplasia are quite complicated with various types of suppressor genes playing different roles, which are important at different times in the development of cancers.

2.3.1. *Caretaker genes*

The caretaker genes are critical to the stable growth of tissues by producing products that maintain the stability of the genome. The same caretaker genes typically control the genome of multiple types of tissues and hence caretaker genes are usually not tissue specific [65–70].

Mutations or other changes in caretaker genes may lead to neoplastic changes; however, such changes in caretaker genes are, by themselves, insufficient to initiate the development of a tumor. Neoplasia arises only in a small fraction of cells having full defects in DNA-repair genes.

BRCA1 and BRCA2 are classic caretaker genes with mutations in both associated with an increased predisposition to breast and ovarian cancers; BRCA2 mutations also are associated with an increased predisposition to pancreatic cancer [71]. Both BRCA1 and BRCA2 play a role in monitoring and/or repairing damage to DNA, most likely through the pathway that repairs double strand breaks in DNA and homologous recombination [72]. Mutations in either of these two genes results in unrepaired damage in DNA. In the case of BRCA1, this may be due to the interaction with Rad51, which plays roles in DNA repair and recombination processes [72,73]. Of interest, phosphorylated BRCA1 and BRCA2 and Rad 51 colocalize during the DNA damage response. Similarly, dysregulation or mutations in mismatch repair genes can result in hereditary non-polyposis colorectal cancers (HNPCC) as discussed subsequently.

The ATM gene, associated with ataxia telangiectasia, can be considered as a caretaker gene [74]. The ATM encodes a protein that belongs to a family of protein kinases, and share similarities with the catalytic domain of phosphatidylinositol 3-kinases at its C-terminal region. As shown *in vitro* and in animal models, ATM is a key regulator of multiple signaling cascades that respond to DNA strand breaks. Cellular responses to the DNA damage involve the activation of cell cycle checkpoints, DNA repair and apoptosis. Exogenous carcinogenic agents, or normal processes, such as meiotic or V(D)J recombination may induce the DNA damage. The ATM gene suppresses tumorigenesis in specific T cell lineages presumably through its caretaker function [74].

2.3.2. Gatekeeper genes

In contrast to caretaker genes, gatekeeper genes are those genes that control the growth of tissues and cellular components within tissues. Thus, in their normal control of growth, they may inhibit proliferation, facilitate apoptosis and other forms of cellular death and/or promote terminal differentiation of cells [65–69].

Proliferation is controlled by the cell cycle which is regulated in part by specific signal pathways such as $p16^{Ink4a}$/pRb and associated cell cycle checkpoints (Cdk4-6 and cyclins D), the $p14^{arf}$/mdm^2/p53 regulatory pathway of G1-S, the APC-β-catenin/TCF4, and the RUNX3 pathway [75]. Also, there are interactions among these pathways, e.g., between pRb and p53 [69, 70].

It is conceivable that gatekeeper genes may be rather tissue-specific and that the concept of gatekeeper genes applies to certain but not all tissue types. For instance, the RB, APC, RUNX3, NF1, MENI, PCT, interleukin 12, SMAD4 and VHL genes are likely to be gatekeeper genes in their respective cell types [7,8,73–78], but ras genes, BRCA1 and BRCA2 are not [65]. It is likely that gatekeeper genes could serve as useful tumor biomarkers because they are somewhat more tissue specific. For example, $p16^{Ink4a}$ is involved in melanomas, pancreatic cancer, and squamous cell oral cancer. APC and RUNX3 are involved in colorectal cancer [75]; SMAD4 is also a gatekeeper in oral cancer [7,8].

2.3.3. Landscaper genes

Landscaper genes are genes that facilitate the growth of neoplastic lesions by creating a microenvironment that aids in unregulated cellular proliferation [77–84]. Based on studies of combining malignant embryonic carcinoma cells from a teratoma with a blastocyst, it is clear that this micro-environment in which tumor cells reside exert the ability to "re-program" malignant cells so that they are controlled and act as normal cells [83]. Similarly, stroma may induce neoplastic changes [78] in benign cells.

The interaction between stroma and epithelial cells is mediated by multiple components. Of these, the extracellular matrix (ECM) plays a very important mediating role [81]. Signals may be transmitted between different cells via their direct interactions with the ECM, as well as via paracrine signals which can be modulated by the ECM which contains proteolytic molecules which can modulate and/or destroy signal molecules. Thus, the ECM acts to control unread signals of a neoplastic phenotype [77–81]. Additional functions that landscaping genes may perform is a general inhibitory modulation of multiple types of signals needed for growth [77–82]. The subsequent discussion of the microenvironment's interaction with neoplastic processes is in the section of "microenvironmental influences in the development of neoplasia". Loss of components of the ECM may lead to a microenvironment which can stimulate unregulated growth, clonal proliferation, and ultimately neoplastic lesions [77–83].

2.3.4. Deficits in mismatch repair or microsatellite instability

An important caretaker gene system is the mismatch repair system that functions to remove mismatched nucleotides and repair the sites of replication errors. A complex of MSH molecules recognizes the mismatched nucleotides and MLH1/PMS2 and MLH1/MLH3 complexes are involved in attachment, removal of the mismatch and repair. Overall, MSH2, MLH1, MLH3, PMS1, PMS2, MSH3 and MSH6 are involved in detection-excision and repair of mismatched nucleotides.

When there are mutations in MSH2, MSH6, MLH1 or PMS2 or loss of expression of MSH2 or MLH1 caused by, for example, methylation of the promoters of these genes, tumors may develop in the brain, colorectum, endometrium or ovary. The loss or dysregulation of mismatch repair in a patient frequently is referred to as a mutator phenotype [84]. Associated with these tumors are microsatellite changes at many positions throughout the genome. Microsatellites are composed of multiple adjacent copies of mono-, di-, tri- or tetra- nucleotides within the DNA. In microsatellite unstable cancers, the runs of nucleotides may be of different lengths than are the same microsatellite runs in germline DNA. Cases with mismatch repair are des-

ignated as having microsatellite instability or replication error rate phenotypes [84–88]. The alterations in the length of simple repetitive sequences of nucleotides have been found associated with a distinct mechanism underlying carcinogenesis [85]. Specific microsatellite markers are found in a broad variety of tissues undergoing long standing continuous damage, inflammation and repair, (LOCDIR), in pre-invasive neoplastic lesions and in tumors, while other microsatellite changes appear to be tumors specific [4,86]. It is hypothesized that MSI is caused by a failure of the DNA mismatch repair (MMR) system to repair errors that occur during the replication of DNA. Failure of MMR is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome [87,88]. This hypothesis is supported by the study of HNPCC (hereditary nonpolyposis colorectal cancer) tumors and other non-hereditary colorectal tumors harboring MSI.

The loss of specific mismatch repair enzymes and the subsequent failure of MMR causes changes in multiple critical genes including TGFβR2, Bax, caspase 5, β catenin, PTEN, APC and β microglobulin [86]. The changes in these and similar molecules lead primarily to hereditary non-polyposis colon cancer (HNPCC), but other tumors also occur in this setting. Of interest, HNPCC is not associated with large numbers of polyps, occurs primarily in the proximal (right) colon, and tends to be a less aggressive lesion than sporadic colorectal tumors [14,89].

2.3.5. The effects of epigenetics on neoplasia

Epigenetics refers to changes in the expression of genes that can be inheritable, but the changes in gene expression are not related to the base sequence of DNA. For example, biochemical changes determine the part of the genome which will be transcribed. This is partially controlled by the nucleosome and the interaction of the nucleosome with chromatin structures during transcription. Specifically, histones of DNA may be modified (e.g., acetylation) affecting heterochromatin of the DNA and hence the ability to transcribe areas of the genome [90–92].

Another major point of epigenetic control is the ability of cytosines that are 5to guanosines, i.e., in CpG group of nucleotides to be methylated by a DNA methyltransferase enzyme. The methylation of CpG islands also causes various degrees of transcriptional suppression. Of interest, about half of the genes in the genome have promoters with CpG collections called CpG islands. Methylation of CpG islands together with histone patterns can act in gene silencing via methylation of CpG groups in promoters and via the formation of heterochromatin [90–92].

Thus, the methylation of cytosine in CpG islands of DNA is an epigenetic process that may alter the function of genes without changing the base sequence of the DNA. The ability of methylation to block transcriptional activation is well documented, particularly within CpG-rich promoters [93]. These types of promoters are known to be methylated at multiple sites, which may result in the inhibition of transcription or gene silencing. In cancer cells, some genes such as VHL and p16, have been found to be methylated and silenced, but only when exhibiting a wild type sequence [94]. Also, silencing of the MLH1 mismatch repair gene by DNA methylation has been detected in colorectal tumors [95]. Because it is unlikely that the mutational status directly could affect the occurrence of methylation, it was speculated that a low level of random methylation could produce the gradual methylation of CpG promoters whenever there is a wild type sequence exposed to constant selection for its progressive transcriptional silencing. This could also occur at sites of tumor-suppressors.

Hypermethylation of promoters may be frequent in some types of tumors, but is usually infrequent in normal tissues. Belinsky and colleagues [96] observed aberrant methylation of p16 as an early event in lung cancer and they proposed it as a potential biomarker for early diagnosis of lung cancer. Ongoing studies may elucidate whether aberrant methylation of p16 is common for other types of neoplasia. The Cairns laboratory found that 93% of 100 kidney tumors demonstrated promoter methylation in at least 1 of 10 genes evaluated. All grades and stages of kidney tumors were affected, but none of 15 normal kidneys or ureteral tissues demonstrated promoter methylation of any of these 10 genes [97]. The pattern of promoter methylation seems to vary with tissue type, so this pattern might be tissue specific enough to be a useful tumor marker [98,99]. Current studies also suggest that the patterns of promoter methylation might be useful in predicting responses to chemotherapy or chemoprevention [100,101]. The discovery of numerous hypermethylated promoters of tumor-suppressor genes, along with a better understanding of gene-silencing mechanisms, underscores the importance of epigenetic mechanisms in tumor development and discovery of new biomarkers [100–105].

2.3.6. DNA/protein correlations

DNA may be prevented from directly being converted to equivalent protein levels via multiple pathways/mechanisms. In some cases, the mRNA may be very stable and only a few copies of mRNA are transcribed from the DNA. In some such cases, the amount of protein available to cells may be controlled by metabolism of the protein. An example of this is $p27^{kip-1}$ which is primarily regulated post-transcriptionally by Skp-2; thus, these two proteins are usually inversely present in tissue [106,107]. Alternatively, an mRNA may be very unstable and may be degraded rapidly. We know little about any mRNA with half life of a few minutes. Also, for some proteins to be functional, requires post-translational modification by, for example, the addition of sugar moieties. In addition, genes may be transcribed alternatively via various forms of splicing. An interesting example of such splicing is CD44 which has many splice variants of which CD44v6 is important as a marker of stem cells in breast cancer [108]. Similarly, splice variants of other genes such as BRCA1 may modulate the function of these genes [109].

2.3.7. Modulation of mRNA

Recently, it has been recognized that the translation of a large proportion of mRNA molecules can be inhibited by single, small, non-coding RNA molecules that are composed of 21–24 nucleotides and that are called microRNAs [110,111]. MicroRNAs (miR) are found in both plants and animals. In animals, the miRs partially base pair with the target mRNA speeding up its degradation [112,113]; however, miRs may less frequently act to inhibit the degradation of the target mRNA. Patterns of activity of miRs have been identified that distinguish between types of cancer and to identify more or less aggressive cancers [114]. For example, loss of miR-133a and gain of miR-224 has been associated with the progression of colorectal cancer and miR-145 was reported to be down-regulated in metastatic colorectal cancer [115]. Similarly, in prostate cancer (PCa) the expression of miRs separated PCa from benign prostate hyperplasia (BPH). Also, the expression of miRs has been correlated with androgen dependence of samples of prostate cancer [116].

Other methods by which mRs are regulated endogeneously also have been reported, including the binding of proteins to mRNA; however, this form of regulation of mRNA has not be as extensively studied as miRs [117,119], but may be just as important.

2.3.8. Microenvironmental influences in the development and progression of neoplasia (see review in [120])

Non-malignant components of the microenvironment of tumors are critically important as to how a tumor develops. This includes tumor stromal interactions mediated by cytokines, tumor-immune interactions mediated by cytokines and exosomes and vasculogenesis/angiogenesis which are mediated by the cytokines, chemokines and stromal interactions [120]. Many of the genes involved in the effects of the microenvironment on neoplastic processes are designated as landscaper genes.

Tumor-stromal interactions depend upon the neoplastic cells of the tumor and cells associated with the tumor such as inflammatory cells, especially macrophages and fibroblasts and conversion of fibroblasts to a carcinoma-associated myofibroblasts. Each of the components of a tumor communicates with the other components, especially via their molecular secretions including cytokines and chemokines.

2.3.8.1. Tumor associated macrophages

Tumor-associated macrophages (TAM) are very important to the progression and metastasis of tumors. They stimulate angiogenesis via their production of VEGF, interleukin 6 and interleukin 8 and they stimulate tumor growth/invasion via TNFα and matrix metalloproteinases. In general, TAM do not function in the phagocytosis of tumor cells or in the presentation of tumor antigens to T cells [121–127].

2.3.8.2. Exosomes

Exosomes, the 40–100 nm vesicles secreted by various cells including the cells of tumors, are part of a pathway by which tumors communicate with and modulate the immune system [128–131] and potentially other tissues. The exosomal vesicles contain both cellular waste molecules and signal molecules. When exosomes fuse with target cells their signal molecules drive target cells to specific actions [128–131]. Signal molecules include TNFα, TGFβ, mRNAs and microRNAs. The suppression of the immune system has been monitored via decreasing the il-2 dependent proliferation of NK and T cells, blocking of T cell activation, induction of T regulatory cells and blocking of the maturation of dendritic cells [132–134].

With the blocking of dendritic cell maturation by exosomes and with the decrease by TAMs in antigen presentation, the ability of the immune system to respond to tumor antigens would be greatly reduced.

2.3.8.3. Myofibroblasts

The fibroblasts associated with the stroma of tumors are activated and become myofibroblasts; these produce vimentin and smooth muscle actin. Thus, these fibroblasts are referred to as tumor associated myofibroblasts (TAMF). TAMFs are similar to myofibroblasts associated with wound healing. Cells mimicking TAMFs may also be produced by epithelial-to-mesenchymal transitions (EMT) of neoplastic cells.

TAMFs contribute to the malignant phenotype in multiple ways. This may include direct contribution by conversion of adjacent uninvolved epithelium to cancer [135,136] and by indirect effects via the secretions of landscaper genes such as S100A4, il-6 and il-8 which may potentiate cellular motility, induce angiogenesis, and modify the stromal environment [137–139]. TAMFs and TAMs also secrete CXCL12, TGFβ, and MMP-13 [140–142]. CXCL12 stimulates the proliferation of some types of tumors (e.g., prostatic carcinoma [141]) and potentiates their metastatic spread [142,143]. The CXCL12 –CXCR4 interaction has varying effects depending on the type of prostate cell. In general, this interaction results in increased stromelysins 1, 2 and 3 as well as matrix metalloproteinases (MMP) [142]. CXCL12 also is a chemoattractant for leukocytes and hence contributes to inflammation which affects the progression of some types of cancer.

2.3.8.4. Angiogenesis

Angiogenesis is one of the 6 original hallmarks of cancer [18] in that it is required to provide nutrients and oxygen to cancer cells which permit growth and survival of cancer. There are multiple factors which modulate angiogenesis – both stimulatory and inhibitory factors of which VEGF (VEGFA) is one of the most studied. The stimulatory factor, VEGF, and its splice variants act by binding to the VEGFA receptor [144–146] to increase the migration and rate of proliferation of vascular endothelial cells; it also creates openings in blood vessels to increase vascular permeability and causes vascular lumens to develop. Most cancers including breast cancers express VEGF and its receptors, KDR or FLT1, and the extent of its expression correlates positively with a poor outcome and more rapid recurrence. Also, VEGF may act to block the beneficial effects of drugs on cancers [147,148].

VEGF like many angiogenic factors is stimulated by hypoxia via a complex of HIF1 α with HIF1 β. il-6 and il-8 also are stimulated by hypoxia as well as levels of glutamine [139]. IL-8 is released via stimulation by NFkB and Activating Protein 1, it then acts as an angiogenic agent similar to VEGF [149–153]. Other angiogenic agents include TGFα, bFGF, angiogenic transcription factor 1 (ETF-1), interferon inducing protein 10 (IP-10), and CXCLI [144,146,154,155].

Of interest, clones may develop without chromosomal or genetic instability. This has been demonstrated by studies in which clones of cells are grown via multiple passages after they have exhibited growth that is non-contact inhibited. After 20 or so generations of passage of non-contact inhibited cells, but not of low density passage cells, the cells can produce malignant tumors when transplanted into a syngenic mouse. This transformation of a clone to a frankly malignant tumor is because the clone has taken advantage of its growth advantage as well as has adapted to its microenvironment. This clonal growth advantage may be via autocrine stimulations in which the group of cells now has established one of the hallmarks of cancer – the development of a continuing self-stimulatory environment [18]. Upon transplantation into a syngenic animal, this self stimulatory environment continues and in addition, the clone may develop autocrine, paracrine or exosomal pathways of stimulation or may be stimulated by the components of the surrounding microenvironment.

2.3.9. Clonal selection

A clone of cells, by definition, is a group of cells growing together that have arisen from a single cell, usually considered to be a stem cell, that has developed secondary to a growth advantage to both the parent and offspring cells. Some of the characteristics of the cell of origin are uniformly carried by the offspring cells which permits the identification of the group of cells as a clone. Clones of cells are most easily detected in tissue when the members of a clone carry a marker related to the transformation such as a mutated p53 [10, 17] or when they have a marker which is a physiologic characteristic such as increased proliferation that is not contact inhibited. Clones in tissue tend to be relatively small, usually with less than 1000 cells unless they form a pre-invasive neoplastic lesion.

Clonal development is thought to be one of the main factors in the development of neoplastic lesions. First, there is either a change in the cells of the clone or a change in the microenvironment. In response to either type of change, there is a growth advantage to the members of the clone. As the clone expands, genetic and epigenetic changes add heterogeneity to the clonal members. This is typically followed by further

selection and evolution until the hallmarks of cancer develop [18].

Most, if not all cancers are monoclonal, but not all actively growing clones of cells are characterized as pre-invasive neoplastic lesions. Because monoclonality is often a marker of evolving neoplasia, it can be used to detect new neoplastic lesions or monitor evolution of the pre-existing tumors. As clones continue to expand, genomic instability may develop; this might be followed by the development of pre-invasive neoplasia depending upon clonal characteristics [156–162].

2.3.10. Viral insertion

Integration of a viral genome into a human host cell can lead to a variety of interactions with their host cells that may be relevant for the induction of cellular transformation, the maintenance of a transformed phenotype, and tumor progression [163]. Identification of novel viruses associated with early stages of neoplastic transformation could lead to new biomarkers and more effective preventive strategies. Several DNA and RNA viruses have been implicated as causative agents or cofactors in certain forms of human cancer. For example, the association of human papilloma viruses (HPV), hepatitis B virus (HBV), and Epstein-Barr virus (EBV) with human cervical, hepatocellular, or nasopharyngeal carcinomas, respectively, has been extensively documented (see [4]). Unlike retroviruses, which are obligate mutagens because their replication cycle and persistence require integration into the host chromosomal DNA, the integration of certain DNA viruses into the chromosomal DNA was once not considered a requirement for viral persistence. This view is challenged, however, by evidence for HPVs integration in most invasive genital cancers, HBV integration in the majority of hepatocellular carcinomas and EBV persistence in integrated form within infected lymphoblastoid cells or Burkitt's lymphomas.

3. Application of biomarkers to understanding neoplasia

Biomarkers may reflect distinct stages of the neoplastic process and could be used in a variety of applications. Potential clinical applications are implicit in the term "biomarkers", which is defined as morphological, biochemical, or genetic alterations by which a physiological or pathological process can be identified or monitored. Potential uses of cancer biomarkers include the following: early detection of neoplastic lesions, distinguishing pre-neoplastic from pre-invasive neoplastic lesions and monitoring patients with established cancer for recurrence, development of metastases or a second primary tumor [164,165]. Further, biomarkers can be used for the assessment of risk for developing cancer and establishing surrogate endpoints for primary or secondary prevention trials [166]. Biomarkers also can be used to predict responses to novel or other therapies. To be clinically useful biomarkers must have high predictive accuracy, and must be easily measurable and reproducible. Tests for biomarkers in most settings must be minimally invasive, and acceptable to patients and physicians. Each of these uses has been discussed in a chapter of this [191].

4. Future directions in the analysis of biomarkers

4.1. Development of highly sensitive and specific biomarkers

It is likely that strategies based on biomarkers could have a great impact on cancer. The challenge is to utilize new scientific and research approaches in order to develop more sensitive and accurate intervention strategies with the greatest impacts on incidence, effective selection of therapies and mortality. In addition, some biomarkers should be detectable early in the carcinogenic process, should be associated with the risk of developing cancer, or the occurrence of precancer. Ideally, biomarkers should be detectable in body fluids and/or in tissue obtained via minimally invasive methods such as fine needle aspiration [167]. Biomarkers of early detection could be utilized to evaluate preventive agents. Any biomarker that is useful clinically should be combined with adequate procedures for quality control to ensure accurate measurement.

Any marker to be used for population-based studies must be characterized as to sensitivity, specificity, accuracy, reproducibility and positive and negative predictive values. Planning for evaluation of molecular markers in clinical trials must also consider the morbidity and costs that may arise from the follow-up of individuals who test positive [160,168]. The use of highly sensitive but less specific tests may result in a large number of false-positive individuals who require further diagnostic examination. For instance, it is estimated that PSA screening of all males age 50–70 would result subsequent diagnostic and therapeutic procedures costing up to $27.9 billion in the first year, as reported in 1994 [168].

The phenomenon of field effect could be used to evaluate the risk of cancer. Many cancers, e.g., head and neck, develop in fields of primed or affected epithelium. Identifying field changes through the application of biomarkers could represent a very early stage of detection; however, if a pre-invasive neoplastic lesion or an SCC develops in the oral cavity, the field effect is established. Also, either the whole oral cavity would require preventive therapy or a watchful waiting approach could be taken until pre-invasive neoplastic lesions develop which could them be treated individually. As new, sensitive technology evolves and our understanding of pre-invasive neoplasia improves, future research should focus on the development of new biomarkers to evaluate the risk in less accessible organs. For instance, predisposition to cancer in non-accessible target organs, such as the lung, might be evaluated by determining the genetic changes that occur in more readily accessible organ sites with which it forms an anatomic and/or a functional continuum that has been exposed to similar carcinogens [165] such as the buccal mucosa. Recently Roy et al., using light scattering technology, has been able to identify colorectal neoplasia based on field effect changes that are caused by cancers and modify the light scattering patterns of uninvolved epithelium [169–171]. Importantly, this approach has been used to identify proximal neoplastic lesions by light scattering patterns in the distal rectum [172]. Thus, this approach, though not understood, may represent a great advance in screening for colorectal cancer.

Of great value for early detection and risk assessment of various malignancies would be the identification of markers detectable in body fluids and various specimens other than tissue, for example sputum, saliva, urine, stool, blood and breast nipple aspirates. Identification of tumor markers –both DNA, RNA and protein based– in body fluids suggests the possibility for developing markers for large-scale screening and risk assessment. To detect DNA-based markers, derived from tumor cells, highly sensitive methods are needed, because tumor cells are greatly outnumbered by normal cells and their products in blood, stool, saliva, and other potential assay targets. It seems, however, that DNA-based markers are less applicable in blood-based screening tests for early lesions [173], because circulating tumor-derived DNA has been generally found associated primarily with advanced tumors. In contrast, proteins detectable in serum and other body fluids might be useful markers for the detection of localized lesions, including early neoplastic lesions. Ideally, biomarkers should be easily measured in body fluids that may be accessible for multiple longitudinal sampling. There is a great need to improve early detection as well as to identify prognostic biomarkers; this includes the identification of biomarkers associated with malignant transformation, invasion, and progression. Such biomarkers also could aid in the characterization of responses to prevention including chemoprevention.

Proteomics-based approaches to detect biomarkers have some distinct advantages over DNA and RNA-based techniques in that they give direct evidence of abnormal gene expression at the time of sampling. In addition, some biomarkers as discussed are regulated post-transcriptionally and hence cannot be detected at the mRNA level; specifically, microRNAs offer a new potential group of biomarkers that currently are being evaluated. Research based upon DNA, RNA as well as protein should be complementary. Identification of DNA sequences coding for specific proteins can stimulate subsequent phenotypic analysis of proteins in tissues or biological fluids.

Approaches to the development of cancer related biomarkers suitable for clinical application have been fragmented and sporadic, resulting in data of limited practical value. Usually the results of studies of biomarkers published in the literature cannot be generalized to the population as a whole. They usually are not performed in defined populations, nor are they prospective. Systematic studies designed to improve sensitivity, specificity and high throughput of cancer related biomarkers have been rather limited until recently. A considerable barrier which has prevented research in validation of biomarkers has been the limited availability of good quality tissue specimens from early pre-invasive neoplastic as well as metastatic lesions, along with respective body fluids and demographic and clinical follow up data. The Early Detection Research Network (EDRN) has established multiple collections of specimens of bodily fluids via which potential biomarkers can be tested. These samples are available to both EDRN and non-EDRN investigators (see the EDRN website, http://edrn.nci.nih.gov/).

4.2. Performance characteristics of biomarker assays

The systematic approach to the identification and development of biomarkers entails several important steps. First, a laboratory performing the study must be able to reliably measure biomarkers in specimens accessible to oncologists and epidemiologists. Biomarker assays that are performed should detect the specific abnormality in a high proportion of cancer patients (it

Table 4
Requirements for Sensitivity and Specificity for Assays

Test	+	−	Total
Cancer	99	1	100
Non-cancer	99	9801	9900
Total	198	9802	10,000

with prevalence = 1% or 100/10,000 cases.
sensitivity = 99%, specificity = 99%.

must be sensitive), but in very low proportion of non-cancer control individuals (it must be specific). Finally, a reasonably large and representative cancer and control group should be available for evaluation to allow precise interpretation of test results and avoid bias [165, 166,174–176]. The selection of an appropriate control group may be difficult without introducing bias. Optimally, the same number of cases and controls should come from each site and the various conditions of sampling should be very similar. Avoiding bias has become much more important as the use of multiplex methods has increased because as the number of measurements in assays increase, the effects and likelihood of bias also is increased [175–177]. Methods using mass spectrometry are especially prone to bias because individual multiple peaks of a complex spectrum may constitute a measurement [176,177].

The selection of molecular features as biomarkers of early cancer will depend on their performance characteristics, which include accuracy, sensitivity, specificity and reproducibility. To be suitable for screening for early detection of neoplastic lesions (asymptomatic and/or high risk subjects), biomarkers must be able to detect disease when it is present, and to identify those patients without the disease (Table 4). Ideally, biomarkers must have both a high sensitivity and high specificity. The sensitivity and specificity should be balanced to increase specificity and to avoid large numbers of false positives. This is a major consideration when biomarkers are used to screen a population with low prevalence of disease in which a positive test can trigger invasive or very costly investigations. A reference value may be chosen to balance the sensitivity and specificity of a biomarker-based test that is based on the prevalence of the disease in the population. Because the prevalence of most cancers is less than 1%, a sensitivity of 99% and specificity of 99% for a single assay is necessary to ensure that false positives do not exceed the number of the positives.

Only cancers with prevalences of greater than 1% will have less false positives than true positives even with sensitivities and specificities of 99%.

One approach to achieve a satisfactory cut-off or reference value for a screening test is to use a receiver operating characteristics curve (ROC); this is a plot of the true positive rate (TPR) versus the false positive rate (FPR). The curve provides the ability to establish cut-off values in which only values greater than or equal to the cut-off values are called positive. For each pair of cut-off values, one can estimate the false positive rate or 1-specificity and the sensitivity or true positive rate.

Both retrospective and prospective studies have to be performed to validate promising markers for clinical usefulness. Retrospective studies that utilize archival pre-invasive neoplastic lesions, such as dysplasia or in situ carcinoma, may be used to determine the presence and frequency of a given molecular alteration in early stages of the neoplastic process. This can be the first step toward ultimate validation of clinical utility. Markers identified in pre-invasive neoplastic lesions are good candidates for further characterization and for evaluation for their effectiveness in detecting cancer at an early stage. Prospective randomized studies may determine whether a promising marker, which recognizes pre-invasive neoplastic lesions, is specific and sufficiently sensitive to accurately predict the development of cancer and lead to improvement in cancer-specific mortality.

A biomarker of an intermediate endpoint in the development of neoplasia can be validated within a prevention (e.g., chemoprevention) trial. Optimally, both a valid and useful marker would be modulated in the short term, e.g., one year, and that modulation might correlate significantly with reduction in cancer incidence in long term, e.g., five to ten years. Such performance standards would validate a marker as an indicator of a clinical and histological outcome for a given type of tumor and a specific drug, but may not be generalizable to other populations at risk or other drug classes. It has been suggested that a panel of biomarkers which are associated with the intermediate points of carcinogenesis may be more useful for determining the efficacy of chemoprevention than any single marker alone [178,179]. As the number of molecular markers increases, complex statistical issues arise which translate into practical clinical considerations. If a large number of markers are tested, the probability that any one is positive in a normal person increases causing frequent false-positive associations. Such problems must be considered and corrected statistically. Examples of markers that are more likely to be useful for a wide variety of uses (e.g., surrogate endpoints, prognosis, early detection) are markers of proliferation and apoptosis.

4.3. Clinical validation

Although many biomarkers for early detection of neoplastic lesions and assessment of cancer risk appear promising, no marker has the needed sensitivity and specificity to identify early neoplastic lesions, to predict cancer risk or to predict responses to chemopreventive agents [180,181]. To aid in biomarker validation, studies should be incorporated into prospective clinical trials. Marker validation in the chemoprevention setting should begin with controlled clinical studies, new or ongoing, consisting of a distinct type of neoplastic lesion and a specific treatment. The feasibility of biomarker evaluation should be based on several criteria, which include 1) differential expression of the biomarker in normal high risk tissues versus pre-invasive neoplastic tissues versus cancer; 2) ability to analyze the biomarker in small tissue specimens; 3) quantitative levels of the phenotypic expression of the biomarker correlating with distinct stages of carcinogenesis; and 4) availability of clinical and preclinical data supporting the modulation of biomarkers induced by the study agent. As suggested by Lippman [178], the evaluation of a surrogate endpoint within chemoprevention trials should consist of several stages including a non-randomized short-term trial in a high risk population with or without pre-invasive neoplastic lesions to determine the feasibility of the study and to select or prequalify candidate markers. The use of a high risk population in early prospective studies is recommended in order to have a sufficient number of expected events in a relatively small sample size of subjects.

In a second step of a validation process, a nontoxic dose and schedule trial should investigate modulation of biomarkers of intermediate endpoints to further define supportive preclinical and epidemiological data and to improve the rational for the third step of validation. Ultimately, the third step of validation should be a long-term phase III trial using cancer incidence, a true endpoint, as the endpoint. It is expected that this step of validation could demonstrate a strong association between modulations of the biomarker and the risk of developing pre-invasive neoplasia and ultimately, cancer [182].

Similarly, the steps in a validation trial for a marker(s) of early detection have been described by Pepe et al. [183]. These are divided into 5 phases which should be completed before a biomarker for early detection is used clinically. These include 1) Preclinical Exploratory Studies; 2) Clinical Assay Development for Clinical Disease. For an assay to be considered to be promising, this phase should distinguish patients with cancer from those without cancer. This phase does not detect early cancer because the samples from patients with cancer are from patients with established cancer; 3) Retrospective Longitudinal Study – This study should use samples obtained prior to diagnosis of either cancer and no cancer (control) subjects. Control subjects are defined as having cancer related subject characteristics and to be cancer free during a specified time of follow-up; 4) Prospective Screening Studies – The goal of phase 4 is to determine the effectiveness of the screening test prospectively in a chosen population. Of importance, is determining the rate of detection and the false referral rate (false positive rate); 5) Cancer Control Studies – This final study is to demonstrate that use of the test reduces cancer mortality via the early detection of cancer. Successfully completing steps 1 to 5 are likely to ensure that such a test will be used clinically. It is likely that the process of population-based screening and intervention will evolve through gradual refinements and improvements of technologies and better analytical tools for basic, clinical and epidemiologic information. Step 5 is in general beyond that necessary for approval by the FDA.

There have been several larger studies which have included or will include components to determine the effectiveness of biomarkers in detecting cancer such as the Prostate Cancer Prevention Trial. Most similar trials have had to overcome difficulties with obtaining appropriate samples from cases and controls. Several approaches and technical developments should aid in future studies. There are now several resources to aid in studies of biomarkers in early detection: First, the PLCO is providing longitudinal samples of serum from patients who have developed a specific cancer. These samples can be very useful in studies focused on Early Detection (http://prevention.cancer.gov/programs-resources/groups/ed/programs/PLCO/about#top). Second, the EDRN has established a collection of samples of serum and sets of aliquots of plasma from cases and controls related to various cancers. These samples are available and provided as unknown de-identified sets of samples for Phase 2 studies of biomarkers. After an investigator completes the analysis of a set of samples, the investigator returns the results to the EDRN which evaluates the results as to whether or not the biomarker is promising as a clinical marker (http://edrn.nci.nih.gov/about-edrn/).

4.4. Technology for biomarkers development

Even the most promising new markers have been limited by technical difficulties and the probable high

cost of implementing their use. The ability to lower cost and improved efficiency can greatly accelerate testing of new generations of biomarkers in screening settings in which lower costs and automation are highly desirable.

Several new technical breakthroughs not only will aid in the conservation of existing samples from cases of cancer and controls, but also will expand the utilization of new types of samples. These include multiplex immunoassays, tissue microarrays, TaqMan Low Density Arrays (TLDA) for real-time quantitative – polymerase chain reactions (RT-Q-PCR), hybridization chips for expression of single nucleotide polymorphism (SNP) and DNA sequencing. Current multiplex immunoassays permit the analysis of many antigens concomitantly on a relatively small sample (100 μl) of serum, plasma or urine. Similarly, small samples of tissue, e.g., fine needle aspirates (FNA) may be homogenized and multiple antigens or genes can be analyzed concomitantly on the same sample.

TLDA-RT^2-Q-PCR analysis also permits multiplex analysis of large numbers of genes on multiple samples. For typical TLDA analyses one can analyze 48 genes including housekeeping genes on 8 samples, 96 genes on 4 samples, 192 genes on 2 samples or 384 genes on 1 sample. Also, it has been demonstrated that by using small primers of less than 100 base pairs, degraded RNA can be measured. This permits RNA to be measured using RT^2-Q-PCR in formalin fixed paraffin blocks; the results are equivalent to measuring the RNA in matching frozen tissue [184,185]. For example, this is the approach by which the aggressiveness of node negative breast cancer subtypes is measured by the Onco*type* DX® test [186–188]. The development of Onco*type* DX® test is a good example of how various tests are now finding their way into clinical use.

Single nucleotide polymorphisms are genetic variation in single bases of DNA of an individual or a population. In general, there is one SNP per 100 to 300 bases; in general SNPs are excellent geographic markers for DNA in that they are located both in non-coding regions and in regulatory regions. SNPs that cause non-conservative changes in coding may affect the function of genes [189].

New technologies have rapidly developed for measuring SNPs. Some service sites can now analyze 10,000 SNP genotypes per day; throughput is increasing yearly and costs are decreasing. Not only can SNPs be determined, but also the copy number can be measured concomitantly [190].

Automation now permits cost effective and rapid identification of gene mutations, deletions, amplifications, or expression patterns in pre-invasive neoplastic lesions and cancer by direct sequencing. Through simultaneous analysis of the expression pattern of thousand of genes, it is possible to investigate whether the majority of differentially expressed genes are tumor specific or cell type specific, and whether most differences are intrinsic to tumor cells or dependent on the tumor microenvironment. Similarly, this methodology could greatly facilitate the detection of early lesion-specific probes which could, in turn, lead to the development of DNA, RNA, or protein-based assays for large scale, population-based screening.

5. Summary

Studies using testing methods such as cervical PAP screening, mammography, fecal occult blood, or colonoscopy have shown that detection of early cancer can reduce morbidity and mortality. Nevertheless, these established methods as well as the PSA screening test are limited by suboptimal sensitivity and specificity, as well as high costs of screening and/or morbidity and costs in dealing with false positive results. Therefore, it seems reasonable to explore the application of the new molecular-based technologies for earlier and more specific detection of neoplastic lesions and even for assessment of risk, that is, identifying patients who are likely to develop pre-invasive neoplastic lesions or cancer. The determination of risk is necessary in order to institute prevention.

The application of molecular methods in the field of early cancer detection is a dynamic process continually driven by expanding knowledge of carcinogenesis and by the availability of more functional and higher throughput technologies. At the molecular level, the detection of earlier cancer will require a more complete understanding of the evolution and regression of pre-invasive neoplastic lesions and associated molecular and genetic changes.

Research focused on the use of biomarkers in translational studies of cancer suggests that no single biomarker is likely to be useful as a specific biomarker in detecting early cancers, in risk assessment or in determining prognosis. However, the phenotypic expression of single molecular species is still very useful as an aid in pathologic diagnosis of tissue and to identify recurrence following therapies (e.g., prostatic specific antigen). An important development and example in this area is the use of the Onco*type* DX® approach to analysis of the aggressiveness of subtypes of breast

cancer [186–188]. Similarly, as the cost of complete sequencing decreases, the speed-throughput increases, and our knowledge of genetic function increases, the ability to analyze a whole genome to evaluate, for example, the risk of developing a specific cancer or the determination of the aggressiveness of specific tumors become more feasible and likely.

The challenges of earlier cancer detection are multidisciplinary in nature including issues in basic science, the development of technology, the finding of key genetic changes, understanding post-transcriptional regulation of proteins and the statistical analysis of changes in multiple gene functions. Increasing integration of medical knowledge and new advancements in various areas of research, including medical oncology, pathology, molecular and cellular biology, molecular epidemiology, genetics, informatics, physics, statistics, and bioethics provide unprecedented opportunities for discovery and development of biomarkers and for understanding of neoplastic processes. New scientific approaches which utilize genomic technology, proteomics and bioinformatics may afford critically important insights into both hereditary and sporadic forms of neoplasia, and revolutionize methods for early detection of pre-invasive neoplastic lesions, risk assessment and prevention. Integration of technology, science and informatics is a key element for accelerating development and application of biomarkers. A critical aspect for better early diagnosis of cancer is the improvement of detection and characterization of early neoplastic lesions. A primary strategy is to facilitate an ongoing interaction between basic scientists, oncologists, clinicians, pathologists, geneticists, theoretical and applied biostatisticians, epidemiologists and other health professionals, because this is critical for the successful application of new discoveries in molecular biology for earlier detection of neoplastic processes. In addition, biomarkers are likely to play key roles as translational research aids the transition of general medical care to a more personalized approach.

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The biology of incipient, pre-invasive or intraepithelial neoplasia

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Abstract. Invasive tumors (cancers or malignant lesions) typically develop in the setting in which there is the presence of putative non-invasive lesions and the development of these non-invasive lesions frequently precedes the development of cancers. For some organs, such as the oral cavity, cervix and skin, the respective putative pre-invasive lesions can be observed over time and documented to progress to invasive lesions. However, for less readily observable lesions, such as those of the prostate, the progression of the pre-invasive lesions, e.g., prostatic intraepithelial neoplasia (PIN) and prostatic proliferative inflammatory atrophy (PIA) to prostatic cancer are more difficult to document. Thus, for most organ systems, specific pre-invasive neoplastic lesions have been proposed based upon the apparent observations of one or more of the following: 1) microinvasive disease developing from a pre-invasive neoplastic lesion, 2) the general association of the pre-invasive lesion with invasive lesions, 3) the subsequent development of invasive lesions following diagnosis of the pre-invasive lesion, 4) correlations of the molecular features of the putative pre-invasive lesion with the matching invasive lesions, and 5) reductions in the rate of cancer following removal of the pre-invasive lesion. When there are mixtures of pre-invasive lesions with actual cancers in the same case, some of the above specific associations are more difficult to make. Several terms have been used to describe pre-invasive lesions, many of which are now less useful as our knowledge of these lesions increases. It is now commonly accepted that these lesions are a features of the spectrum of neoplastic development and most are accepted as "neoplastic lesions" with associated molecular features, even though they may be reversible even if they have mutations in suppressor genes (e.g., p53) or are associated with viral etiologies (e.g., cervical intraepithelial neoplasia). The overall term, "pre-invasive neoplasia", seems to best describe these putative pre-invasive lesions. Thus, terms such as incipient neoplasia should be abandoned. The term "intra-epithelial neoplasia" with an associated grade, which has been developed for pre-invasive neoplastic lesions of the cervix, i.e. cervical intraepithelial neoplasia (CIN), seems to be a terminology that adds consistency across epithelial organs. Thus, adoption of these terms for the additional organ sites of pancreas (PanIN) and prostate (PIN) seems accepted. Less descriptive terms such as the degrees of dysplasia of the oral cavity and bronchopulmonary system and actinic keratosis and Bowen's disease of the skin might be better designated as oral intraepithelial neoplasia (OIN), pulmonary intraepithelial neoplasia (PulIN) and dermal intraepithelial neoplasia (DIN). The etiology of pre-invasive neoplasia is the etiology of the matching cancers. Some obvious initiating factors include exposure to the whole range of ionizing and non-ionizing radiation, tobacco abuse and a broad range of other carcinogens (e.g., benzene). A frequent initiation factor is the setting of long standing continuing damage, inflammation and repair (LOCDIR) which leads to early molecular features associated with neoplasia after about one year. An excellent example of this is ulcerative colitis (UC) in which dysregulation of microsatellite repair enzymes have been documented one year following diagnosis of UC. While the nomenclature, description, diagnosis and etiology of pre-invasive neoplasia has advanced, approaches to therapy of such lesions have not progressed adequately even though it has been identified that, for example, removal of polyps periodically from the colorectum, DCIS from the breast, and high grade CIN from the cervix, results in a reduction in the development of cancers of the colorectum, breast, and cervix, respectively. With the development of more molecularly targeted therapy with fewer side effects, preventive therapies may be more successfully targeted to pre-invasive neoplastic lesions.

Keywords: Intraepithelial neoplasia, pre-invasive neoplasia, prostatic intraepithelial neoplasia, pancreatic intraepithelial neoplasia, cervical intraepithelial neoplasia, adenomatous polyps, ductal carcinoma in situ, lobular carcinoma in situ, inflammation, radiation, viral infections, carcinogens, dysplasia, actinic keratosis, repair, angiogenesis, LOCDIR

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1. Introduction

Malignant (invasive neoplastic) lesions are thought to develop from specific "pre-invasive neoplastic lesions" whose histopathologic features have been described for most epithelial organ systems. Sometimes these lesions are referred to as incipient, pre-cancerous, or pre-neoplasia; however, for many epithelial organ systems such lesions are now referred to as intraepithelial neoplasia or, less specifically, e.g., for the oral cavity, as epithelial dysplasia or pre-invasive neoplasia. The characterization of these lesions as "neoplasia" is justified because about 30% to 60% of the most aggressive of these lesions are thought to progress to malignant (invasive) lesions. Also, these lesions have specific molecular changes which separate them when compared to uninvolved epithelial areas [1–3]. In addition, the higher grade intraepithelial lesions usually have molecular changes which are different from lower grade intraepithelial lesions.

For some organ systems such as the breast, even earlier histopathologic lesions thought to lead to pre-invasive neoplasia (i.e., the pre-invasive lesions of ductal carcinoma in situ [DCIS] or lobular carcinoma in situ [LCIS]) have been identified. These have been designated as lobular or ductal atypical hyperplasia, LAH and DAH respectively. Although patients with LAH and DAH as well as even earlier lesions described as hyperplasia or fibrocystic changes carry an increased risk of developing mammary carcinoma [4], these lesions are not classified as neoplastic. In the breast, DCIS and LCIS are divided into low grade and high grade lesions. In other organ systems such as cervix, prostate and pancreas, intraepithelial neoplasia also is graded, i.e., intraepithelial neoplasia 1, 2 and 3, with less aggressive lesions in the 1 category. For example, for the prostate we refer to one type of pre-invasive neoplastic lesions as prostatic intraepithelial neoplasia with grades PIN 1, PIN 2 or PIN 3 with PIN2 and PIN3 referred to as high grade PIN to indicate the lesions most at risk for progressing to prostatic adenocarcinoma [5–7]. Rarely, in addition to the main intraepithelial neoplastic lesions hypothesized to lead to invasive neoplasia, other alternative pathways (lesions) that may lead to invasive cancer also have been identified or proposed. In the case of pancreas, one such lesion, intraductal papillary mucinous neoplasia (IPMN), [8] and in the prostate, proliferative inflammatory atrophy (PIA), have been proposed as alternative pre-invasive neoplastic lesions [9].

Pre-invasive neoplastic lesions typically occur in small areas of an organ; these areas may frequently be multifocal throughout the organ. A good example of this is the skin in which there may be multiple areas of actinic keratosis on the sun-exposed skin (e.g., arms and face). For the cervix, multifocal areas and grades of CIN may arise along the squamocolumnar junction. Each grade of pre-invasive neoplasia usually has a distinct histopathologic microscopic appearance upon which the grade of intraepithelial neoplasia is based.

In some cases, small tumors have been described as adenomas with the view that malignant tumors may or may not develop from these small adenomas. This was once the view, for example, of small tumors of the cortex of the kidney, which if less than 3 cm were once designated as renal adenomas. We now view most such small tumors of the renal cortex as just early forms of renal cortical carcinomas which have not had time to metastasize; thus, because these small adenomas were not associated with metastatic disease, they were initially considered to be benign. However, some tumors such as adrenal cortical adenomas which meet specific histopathologic and biochemical criteria grow very slowly and are still considered to be benign neoplastic lesions [10].

In general, most intraepithelial neoplastic lesions have been identified by their association with invasive or metastatic cancer (guilt by association) or rarely by areas in intraepithelial neoplasia in which there appears to be invasion into stroma from the intraepithelial lesion [11]. In the case of colorectal neoplasia, adenomatous polyps have been identified to be a pre-invasive neoplastic lesion because if all such polyps are removed from the colorectum, patients very rarely develop colorectal adenocarcinomas [12,13]. Similarly effective local treatment of CIN2 and CIN3 of the cervix prevents the development of squamous cell carcinoma (SCC) of the cervix.

2. Spontaneous resolution of pre-invasive neoplastic lesions

The pre-invasive neoplastic lesion is not always committed to progressing to invasive cancer. Specifically, we have noted that areas of dysplastic leukoplakia may spontaneously move geographically in the oral cavity as well as spontaneously partially or completely resolve [14].

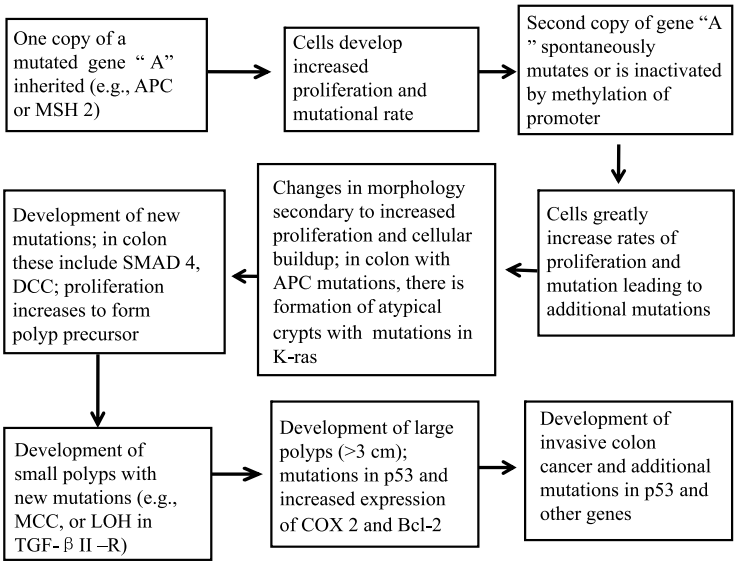

Fig. 1. Describes a general pathway by which a mutation in a single copy of inherited gene such as APC may cause the development of pre-invasive neoplasia of, for example, the colorectum. Mutations in such genes as BRCA1, BRCA2, APC, MSH2 and MLH1 may follow such a pathway in other tissues. Mutations in DNA repair pathways do not typically produce the pattern of large polyps of the colorectum seen in mutations with APC.

Similarly, it is known that if cervical intraepithelial neoplasia–3 (CIN-3) lesions are not treated over a decade, about 25 to 35% will regress. If the initial lesion is moderate dysplasia (CIN2) a larger proportion, 45 to 65%, will regress to normal and the proportion regressing increases in patients who are over 50 years of age (Reviewed in Koss [15]). Whether or not there is potential spontaneous regression of intraepithelial neoplastic lesions in other organs is unknown; however, by analogy this is our guess.

3. Molecular changes in pre-invasive neoplasia

The identification of molecular changes during the development of intraepithelial neoplasia in some organ systems has been possible via analysis of familially inherited neoplasia. Dr. Vogelstein and colleagues [16] developed a model of molecular changes in inherited colorectal neoplasia based on neoplasia that develops from families with familial adenomatosis polyposis coli (FAP). In fact, the first gene identified as being dysregulated in FAP was designated adenomatous polyposis coli (APC); identification of mutations in APC was followed by an understanding of how APC interacts with β catenin and an identification of subsequent mutations or dysregulation of K-ras, DCC, SMAD4, MCC and p53 [15]. Mutations in microsatellite repair genes such as MSH2 and MLH1 also have been identified as the cause of colorectal neoplasia in hereditary non-polyposis colon cancer (HNPCC) [17]. Figure 1 demonstrates how hereditary tumors may develop based on inheritance of a mutation in a single gene.

In general, some of the same mutations that occur in familial cancers occur in sporadic cancers, but such mutations may not occur in the same temporal order. Nevertheless, understanding the specific dysregulation of genes and the pattern of genetic dysregulation of familial cancers has provided important insights to the development of genetic dysregulation in sporadic cancers; thus the genetic changes in cancers in individuals from families with FAP and HNPCC follow a sequence that is useful in understanding inherited as well as sporadic colorectal cancers.

Probably in most cases, specific molecular changes may precede the development of discernable histopathologic lesions. In mice exposed to UV-B, mutations in p53 precede discernable lesions by several months [18]. Also, in humans up to 4% of sun exposed skin has been estimated to involve cells with mutations in p53 [19, 20]. Obviously, most of these areas of cells with p53 mutations never progress even into actinic keratoses.

4. Stochastic versus stem cell models in the development of intraepithelial neoplasia

There are two major hypotheses of how preneoplasia develops and progresses – i.e., the stem cell hypothesis

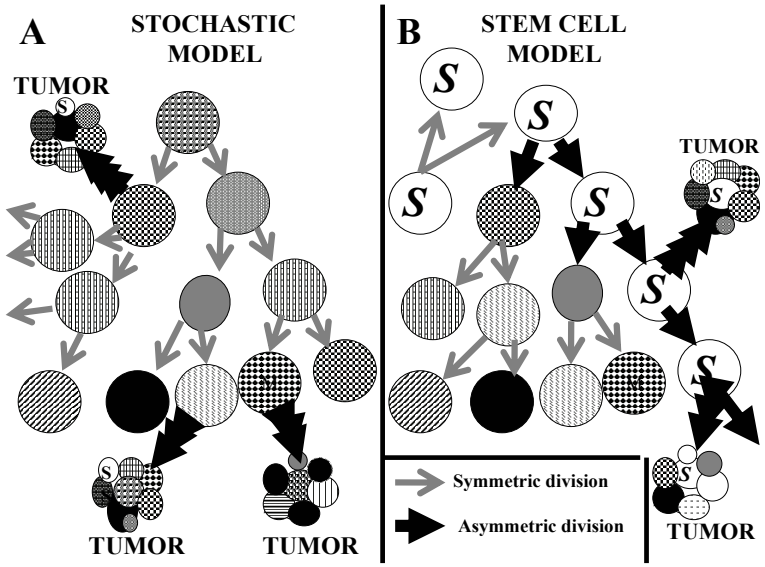

Fig. 2. Panel A demonstrates the model of the stochastic development of cancers. In this model, each child cell is somewhat molecularly different as is indicated by the different patterns of the cells. Note that a tumor may develop from any of the child cells. Most divisions until the pattern of cancer develops are considered symmetric. Multiple tumors are demonstrated to arise, but this is unlikely to occur. Once a pre-invasive neoplastic lesion develops, it is likely to overgrow all surrounding pre-invasive neoplastic cells and hence destroy the rest of the group of cells. Panel B demonstrates the model of how cancers develop from stem cells. In this model, cancers may develop only from stem cells (i.e., cells labelled by an S). While these two models for illustration demonstrate multiple tumors developing from a group of cells, this would be unlikely to occur as discussed above. Note that a stem cell generating two stem cells is considered a symmetric division; in contrast, a stem cell generating a more differentiated cell plus a stem cell has undergone an asymmetric division.

in which preneoplasia develops, only from mutations in stem cells and/or in pluripotent early progenitor cells versus the stochastic model in which preneoplasia develops from any cells in which mutations of specific genes occur.

A stem cell or early progenitor cell is defined as a cell that continually self-renews even while producing a more differentiated cell during the same mitosis. Such mitoses are hypothesized to be asymmetric as to DNA transfer to child cells. Stem cells are described as a small subpopulation ($< 5\%$) of the cells within a tissue compartment which upon removal and transplantation to an appropriate environment will, regenerate the phenotypic characteristics of the original epithelial compartment. Thus, as a stem cell divides, both new stem cells are created while the non-stem cells created on each mitosis differentiate to form the differentiated characteristics of the cells of the tissue compartment. In some cases, one characteristic stem cell may maintain more than one cellular compartment via its differentiated children, e.g., alveolar, ductal and myoepithelial cells of the breast. Typically, each stem cell controls only a small geographic area (niche) of a tissue compartment.

The difference between a stem cell model for the development of cancers and the stochastic model is represented in Fig. 2. In general, in the stem cell model only stem cells give rise to cancers. In the stochastic model, cancers can arise from any proliferative cell with specific mutations. It is likely that after one clone of specifically mutated cells develops, it rapidly overgrows the remaining cells of the compartment no matter which model is valid.

The major evidence for the stem cell hypothesis is that normal tissues and cancers develop preferentially from cells expressing stem cell markers and only a few of these cells are necessary to produce a neoplastic lesion in vivo. Specifically, it takes the implantation of 100,000 or more unsorted cells to grow a xenograft tumor, but implantation of less than one hundred stem cells has been demonstrated to be necessary to produce a xenograft tumor [21]. One of the main consequences of the stem cell model is that to treat cancer and other neoplasia successfully, the therapy needs to be directed at the stem cells of the cancer, as well as the typical cells of the cancer; otherwise, the stem cells that are frequently resistant to most therapies will remain after therapy and the lesion will recur [22–24].

An additional aspect of the current model of stem cells is that the stem cells express a characteristic set of biomarkers; however, these molecular features may

vary with each specific organ system. The stem cells for neoplasias of the breast are perhaps the best characterized; the stem cells of the breast express aldehyde dehydrogenase activity (ALDH) and the ALDH1 protein. These stem cells also are CD44-v6$^+$ and CD24$^-$ and LIN$^-$ [23,73].

In the stochastic model, Fig. 2, Panel 1A, the initial parental cell can be any cell, not necessarily a stem cell. This initial parental cell begins dividing and each of the cellular children is similar to, but different from the parent. As with grandchildren, the subsequent generations are even more diverse than the original parent. At any point, any offspring may generate a "bad seed" which develops a molecular change that leads to child cells with characteristics of a neoplastic lesion. The more proliferative child cells are more likely to develop a new mutation, each new mutation may increase proliferation as well as the mutational rate and decrease rate of apoptosis; ultimately, a pre-invasive neoplastic lesion may develop. In contrast, in Fig. 2, Panel 1B, cancers only develop from stem cells, but the "bad seed" pathway is similar.

5. Environmental exposures and the development of neoplasia

Multiple types of environmental exposures increase the risks for developing specific types of cancers. Broad categories of such environmental exposures are ionizing and non-ionizing radiation, infectious microbiota, chemicals and physical agents such as asbestos fibers, wood dust, soot and rock (silica) fragments. Chemicals may range from ions and elements, especially metals, to complex molecules produced by microbiota such as fungi (e.g., aflatoxin B1), manufactured by humans (e.g., diethylstilbestrol) or produced by changes in dietary components on cooking (e.g., heterocyclic amines).

6. Radiation and the development of neoplasia

Radiation is a major cause of specific cancers. For example, although controversial, the gas, radon, which may accumulate in houses and in mines, has been implicated in the development of cancer of the lungs, especially in smokers [25] with a relative risk 1.8 (1.1 to 2.9). Actually, the most common exposure to radiation that leads to the development of neoplasia is solar radiation of the skin.

Frequent molecular changes are noted in squamous cell carcinoma (SCC) and in basal cell carcinoma (BCC) of the skin. Specifically, there are mutations in p53 in about 70 % of SCCs and 50% of BCCs. Of interest, about 4% of the epidermis of normal appearing human skin from individuals with sun exposure but without cancer contains p53 mutated clones of 60 to 3000 cells in size [20] and the risk of developing SCCs and BCCs is correlated with the extent of mutations in p53 [26]. A mutational pattern in p53 is more common in sun exposed skin than in sun shaded skin (X40). These mutations in p53 are usually C→T or CC→TT [27,28]. For clones of smaller size, the clones were conical with their apex frequently at the basal epidermis suggesting an origin from this area where there are basal cells and/or stem cells [20]. Other studies in mouse models have noted UV-B induced clones of p53 are noted months before the development of skin tumors. Also, based on mouse models, by using UV blockers (SPF-15 sun screens) the occurrence of most p53 mutational clones can be prevented or reversed [18].

In general, primarily UV-B solar radiation and to some very small extent UV-A solar radiations cause p53 mutation in the epidermis. These mutations of suspected stem cells induced by UV-B are thought to be promotional events that lead to clonal expansions of p53 mutated stem cells. These clones then grow, remain static or potentially regress until other molecular changes occur that lead to the development of actinic keratoses and ultimately to SCC.

The major recognized pre-invasive neoplastic lesion of the skin is actinic keratosis which progresses at a low rate to squamous cell carcinoma (SCC) of the skin. Clones of cells with mutated p53 are likely to be the precursors of most actinic keratoses, especially Bowenoid actinic keratosis [28].

7. Chemical effects on the development of neoplasia

A broad range of chemicals can be involved in the development and/or progression of various neoplastic processes. For example, metals such as cadmium, beryllium, arsenic and chromium have carcinogenic potential; however, the methods of action of these metals are not understood. Organic compounds such as cyclic hydrocarbons and cyclic amines have been associated with numerous malignancies. Most have been noted in humans with various environmental exposures; in

Table 1
Chemical carcinogens [32]

Chemical agent	Postulated associated malignancy
Aflatoxin B_1	Hepatocellular carcinoma
Arylamines (e.g., 2 naphthylamines)	Bladder carcinoma
Benzene	Acute myeloid leukemia
Benzidine	Bladder
Diethylstilbestrol	Vagina, cervix
Heterocyclic amines	SCC of the lung
Metals	
– Arsenic	Skin, bronchus, liver
– Beryllium	Lung
– Cadmium	Lung, prostate, pancreas, kidney
– Chromium	Lung
– Nickel	Nasal sinus, bronchus
N-nitrosamines	Gastrointestinal; adenocarcinoma of the lung
Polycyclic aromatic amine	Lung, skin, urinary
Vinyl chloride	Liver

addition, these chemicals have proved to be potent carcinogens in animals. Some potential carcinogens are listed in Table 1; the associated cancers were primarily determined in animal studies.

Many chemicals act via the formation of adducts with DNA which following DNA duplication subsequently cause mutations in DNA. For example, mycotoxins typically react with nitrogens of adenine and guanine molecules. In theory, stem cells are usually the sensitive target with some adducts functioning in initiation and frequently different adducts in promotion of neoplastic lesions. Initiation and promotion are probably sufficient to lead to early pre-invasive neoplasia. Subsequently, mutations occur that lead to progression and to eventual malignancy.

In other cases, the actual chemical must be metabolized to reach its optimal carcinogenic potential. Many human enzymes rapidly metabolize carcinogens to non-carcinogens and are hence protective; other human enzymes modify chemicals to produce optimal carcinogens. These enzymes vary in their potency among individuals due to genetic polymorphisms in the enzymes. Polymorphisms can be identified by analysis of SNPs and may either represent positive or negative risk factors for the development of neoplastic lesions. For example, ethanol is associated with a greater risk of developing colorectal cancer in individuals with the E487k polymorphism of aldehyde dehydrogenase 2 (ALDH2) which results in greatly reduced enzymatic activity of ALDH2 [29]. Similarly, polymorphisms in 5, 10- methylenetetrahydrofolate reductase plus low folate levels increase the risk of colorectal cancer associated with ethanol consumption [30,31].

One of the reasons smoking of tobacco may be so strongly associated with the development of pre-invasive neoplasias and cancer of the oral cavity, larynx, lung, urinary tract, and colorectum are the many (> 50) carcinogens in tobacco smoke, as well as the many different types of carcinogens, (e.g., metals, n-nitrosamine, polycyclic aromatic hydrocarbons). Thus constant smoking is likely both to initiate and to promote the development of pre-invasive neoplasia in multiple organs exposed to these carcinogens.

Other chemicals may affect cancers once the pre-invasive or advanced stages of cancer have developed. For example, copper has been reported to potentiate the progression of cancers [33] via the involvement of copper in the development of angiogenesis and/or other features that affect the development and progression of neoplasia.

8. Microbiota and infections

After the infectious etiology of neoplasia was first identified in birds, viral transmission of various neoplastic processes was confirmed in multiple animals including rabbits and mice. One of the earliest identified neoplastic processes associated with viral infection in humans is cervical squamous cell neoplasia.

Several examples of human neoplasias that arise secondary to viral infections are listed in Table 2. The most recently identified examples of neoplasias arising from viral etiologies include Merkel cell tumor of the skin [34].

The viral etiology of cervical neoplasia is one of the better understood models of viral interactions in humans to produce neoplastic changes in cells. After infection with multiple HPV serotypes especially 16 or 18, there is a dysregulated production of the E6 and

Table 2
Examples of viral causes of neoplasia

Virus	Tumor
Hepatitis V virus (Hepadnovirus)	Hepatocellular carcinoma
Hepatitis C virus (Flavivirus)	
Epstein-Barr virus (Herpesvirus)	Burkitt lymphoma
	Hodgkin lymphoma
	Nasopharyngeal carcinoma
Kaposi sarcoma herpesvirus (HSV-8)	Kaposi sarcoma
	Lymphoma
Genital Human Papilloma Viruses	Anal carcinoma
	Cervical carcinoma
Human T Cell Leukemia Virus Type-1 (HTLV-1)	Adult T cell lymphoma
Polyomavirus	Merkel cell tumor of skin

E7 viral genes. E6 binds and inactivates p53 through ubiquination and it also activates hTERT. E7 binds pRb and associated genes p107 and p139 causing increased cellular proliferation. However, these actions of E6 and E7 are not considered sufficient for the production of cervical cancer.

Once cells are infected with HPV and the viral regulatory proteins bind to p53 and pRb, a cascade of several very characteristic changes occur in the cervical epithelium. One of the first observable morphologic changes is koilocytic changes in the mid-epithelium of the cervix. This change results because of the accumulation of material in the cytoplasm that does not stain with eosin of the hematoxylin and eosin (H&E) stain causing a halo appearance in cells. An area of the cervix with only such koilocytic changes is classified as CIN1. Thereafter, more and more cells of the squamous epithelium of the cervix become less differentiated, leading to various grades of cervical intraepithelial neoplasia. CIN3 lesions are thought to be at greatest risk of progression with at least 1/3 of CIN3 lesions progressing to squamous cell carcinoma if not treated [15]. To aid in deciding which cervical lesions require careful follow, analyses can now be performed to identify infections by the thirteen most common high risk serotypes of HPV infections including HPV16, 18, 31, 33, 39 and 45 which can lead to cancer and HPV serotypes 6, 11,42, 43 and 44 which can cause genital warts. Also, young women are now being vaccinated against specific high risk HPV serotypes including 16 and 18 as well as HPV 6 and 11 in order to reduce the risk and costs associated with HPV infections.

Infection of the gastrointestinal system with the bacterium *Helicobacter pylori* (*H. pylori*) has been associated with the development of gastric carcinomas and/or gastric lymphomas of MALT type. There also is an association of *H. pylori* infections in most cases of chronic gastritis and peptic ulcers. Products of *H. pylori* may also affect tissues downstream of the primary site of infection.

Few other classes of bacteria have been definitely identified or even associated with the development of neoplasia; however, fungal infections that produce toxins such as aflatoxin may cause liver cancer in certain areas of the world. Similarly, infections with *Schistosoma haematobium* cause areas of localized continuing damage, inflammation and repair in the bladder. Such infections may lead to carcinoma of the bladder, especially squamous cell carcinoma of the bladder.

9. Longstanding continuing damage, inflammation and repair in the etiology of neoplastic processes

Many neoplastic processes/lesions develop in the setting of "long term continuing damage, inflammation and repair (LOCDIR)." We define LOCDIR as severe damage to tissue that continues constantly for more than 1 year and which induces continuing inflammatory responses, severe cellular damage and tissue repair. In this setting recognizable molecular and histopathologic neoplastic lesions (dysplasia) may develop, usually after more than 5 years of LOCDIR. We chose 1 year because dysregulation of microsatellite (MS) repair genes have been noted in continuing ulcerative colitis after 1 year's duration while more acute forms of colitis do not have changes in microsatellites [35]. Examples of lesions classified as LOCDIR include ulcerative colitis, Crohn's disease or non-specific colitis leading to colorectal neoplastic lesions and chronic pancreatitis in which pancreatic cancers may develop [36] and Barrett's esophagus caused by acid reflux from which esophageal adenocarcinomas may develop. Similarly, squamous cell carcinoma may develop along chronic draining sinus tracks or in the bladder secondary to *Schistosoma haematobium*. As with most categories of

LOCDIR, dysplastic and molecular changes may develop in the cells of the affected organ. Even actinic damage in specific sun exposed individuals who work primarily outside may be so constant that the condition could be classified as LOCDIR.

Multiple potential mechanisms have been proposed for the development of neoplastic lesions in the setting of continuing extensive damage to cells and resulting inflammation. Continuing damage may cause the release of inflammatory mediators as well as reactive oxygen species (ROS) or reactive nitrogen species (RNS) which can further damage DNA and cause DNA adduct formation. Similarly, DNA adduct formation may result from local carcinogens associated with the inflammation, damage and repair. Our view is that neoplastic changes occur because the severe cellular damage requires extensive repair of the damage with increased proliferation of cells. Increased proliferation in areas of damaged tissues, frequently may lead to molecular changes in cells due to DNA replication in the setting of genomic damage. In the setting of severe LOCDIR, cellular injury also may result in such extensive damage to cellular DNA that the ability of cells to repair the damage to DNA prior to replication may be exceeded; also ROS and RNS may prevent accurate repiair of the DNA [35]. Thus, during cellular replication, mutations and other changes in DNA may develop. As increased numbers of isolated mutations and other changes in DNA develop, the mutational rates of such damaged cells are likely to increase.

An excellent example of LOCDIR with the development of mutations in normal appearing cells is ulcerative colitis (UC) in which molecular changes in DNA can be identified in normal appearing cells in as little as 1 year after the onset of UC [35]. UC is an example of how years of damage and repair may cause the development of pre-invasive neoplasia (dysplasia) and an increased risk of the development of carcinoma within 7 to 10 years after the onset of UC, depending upon the severeity of the UC [37].

One of the pathways that may be affected by LOCDIR is the microsatellite (MS) repair system. When UC mucosa is evaluated for microsatellite changes, MS instability can be detected in about 15% of randomly collected specimens of UC, but not in ischemic colitis which is a short term inflammatory condition [35–37]. Of interest, some loss or changes in MS were found adjacent to the genes, APC, p53 and DCC [38–40].

When changes in DNA including loss of chromosomal areas occur adjacent to major suppressor genes such as p53 or Rb, the changes in DNA may frequently involve only genes geographically adjacent to the major suppressor genes or oncogenes on the affected areas of the chromosome, but not the major suppressor genes or oncogenes. A new concept is that some genes geographically related on chromosomes to the major suppressor genes frequently have a biological relationship to these major suppressor genes. Although these geographically related genes are usually not considered to be major suppressor genes, in the bladder losses or mutations in such genes have been associated with clonal proliferations. These genes have been designated as "forerunner genes" because their initial involvement usually leads to subsequent involvement of the major suppressor genes in their chromosomal area e.g., p53 or Rb [41,42]. Subsequently, the involvement of major suppressor genes would then lead to the development of pre-invasive neoplasia. Alternatively, defective repair of DNA could lead to haplodeficiency of important regulatory genes such as TGFβR2 or TGFβR1 [43–45] and subsequently to pre-invasive neoplastic lesions. Also, some individuals may be inherently more sensitive to such changes because they have inherited SNPs which cause overall inefficient functioning of specific genes.

10. Immunesurveillance and immune responses to early neoplastic changes

The escape of early neoplasia from immunesurveillance has been postulated for decades and this concept has been the driving force for some approaches to immunotherapy which seek to increase the effectiveness of the immune system in the management/control of cancer. As discussed, pre-invasive neoplasia of the cervix, oral cavity, and skin may spontaneously change and may regress over months to years. Also, some invasive lesions, especially melanomas, have been reported to regress spontaneously. It is unknown if and how the immune system is involved in such spontaneous changes [15,46]; however, the immune system is hypothesized to modulate the development and progression of neoplastic lesions (reviewed in Zhang and Grizzle [46]).

Some examples of the more clear indications that neoplasias are under immune suppression are that cancers that are infiltrated by lymphocytes such as medullary carcinomas of the breast and stomach tend to have better outcomes than similar cancers without lymphocytic infiltration and that upon immunosuppres-

sion for organ transplantation, the incidence of non-melanoma skin cancers, thyroid cancer, head and neck cancer, colorectal cancer, as well as bladder, ureteral and renal cancers all increase [47]. Similarly, patients with organ transplants and patients with HIV are more susceptible to neoplastic lesions caused by viral infections (e.g., Kaposi sarcoma, SCC of cervix, vulvar and anus, hepatocellular carcinoma and EBV induced lymphoproliferative neoplasia). Of interest, some molecular changes leading to preinvasive neoplasia may spontaneously resolve if the stimulus that leads to the molecular change is removed. For example, when p53 clones or actinic keratoses that develop in the skin of mice secondary to UV-B radiation are shielded from UV-B radiation, both the size of the p53 clone and lesions of actinic keratosis tend to resolve. However, because the extent of the resolution of UV-B damage does not change in Rag1 knockout mice which cannot develop B, $\alpha\beta$T, $\delta\gamma$T or natural killer T cells [48], this suggests that parameters other than the immune system are at work in the response of these lesions to withdrawal of the causative stimulus. If this lesion were considered to be a LOCDIR lesion, perhaps the stimulus of constant severe repair is lost and cells with p53 mutations and/or chromosomal losses (MSI changes) are deleted by non-immune mechanisms.

Neoplastic lesions are usually recognized by the immune system and the response results in increased acute phase reactants (APR), stress proteins and antibodies to specific products of neoplastic lesions (tumor specific antibodies). Cells of neoplastic lesions may contain numerous non-self antigens including mutated proteins, splice variants of proteins, oncofetal proteins and atypically modified proteins (e.g., proteins with increased glycosylation). When tumor cells die (a very frequent occurrence) these non-self molecules as well as numerous products of injured and dying cells are released into the interstitium and are picked up by the vascular system. Also, some of these "non-self" molecules may be displayed on the surface of neoplastic cells and create an immune response. Similarly, larger cancers may activate the immune system by signals of tissue damage and hypoxia. The increases in APR and tumor specific antibodies have been used as non-specific targets in the early detection of cancers [47] but their use in early detection has been criticized as lacking specificity. Although the immune system seeks to control (inhibit) neoplastic lesions, such lesions in turn seek to suppress the immunomodulatory mechanisms directed at their control. Specifically, myeloid suppressor cells (MSC, $Gr1^+$ $CD11b^+$) are increased in the spleen and the bone marrow of humans and animals with specific types of neoplastic lesions. Also, the accumulation of MSCs correlates with increased burden of cancers and poor survival (reviewed in [49,50]).

It is hypothesized that suppression of natural killer cells (NK) by MSCs is important in suppression of the immune system [51] as is the production of T regulatory cells which maintain tolerance to self-antigens [52]. However, the infiltration of neoplastic lesions by mast cells and other granulocytes and macrophages results in products (e.g., MCP4, MCP-6, tumor associated osteopontin, cathepsin D, il-6, il-8) being released that are mitogenic for neoplastic cells, affect cancer remodeling of tissues and increase angiogenesis – all changes that may cause the growth and metastasis of cancers [74].

Some of the above protective changes induced by neoplastic lesions occur secondary to the secretion of exosomes – tiny (40 to 100 nm) membrane bound particles produced by reverse budding of endosomes – multivesicular bodies. Exosomes are released from cancer cells and other cells into the extracellular space and ultimately a proportion of exosomes are picked up by the vascular system. Exosomes may carry hundreds of common "waste" proteins as well as specific proteins that are used by exosomes from different tissue sources to provide specific signals when the exosomes fuse with target cells [53–55].

Examples of signals provided by exosomes include TGFβ from exosomes from the thymus that affect the induction of regulatory T cells [52]. Similarly, TGFβ and PGE2 in exosomes from tumors promote bone marrow myeloid cells to become myeloid-derived suppressor cells (MDSCs) which promote tumor progression; for example MDSCs produce il-6 and VEGF [56] which may induce stromal development and angiogenesis.

Studies in animals have demonstrated that exosomes produced by tumor cells cause a decrease in NK cells and the cytotoxic activity of NK cells [51]. Exosomes also react with immature dendritic cells ($Gr\text{-}1^+$, $CD11b^+$) and monocytes ($CD14^+$) and inhibit their maturation to mature dendritic cells [57]. Secretion of exosomes also causes the build-up of myeloid suppressor cells in the spleen and in bone marrow as discussed previously [57]. MDSCs can also then suppress NK and killer T cells [50,56].

11. General characteristics of intraepithelial neoplasia leading to adenocarcinoma

Adenocarcinomas develop in multiple organ systems. This includes the breast, prostate, ovary, en-

Table 3
Adenocarcinomas and associated pre-invasive neoplastic lesions

Organ	Pre-invasive neoplasia leading to adenocarcinomas	Principal features of lesions containing dysplastic epithelial cells
Breast	Low and high grade: ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS)	Expanded duct with reduced discontinuous and flattened myoepithelial cells surrounding dysplastic epithelial cells. One type of high grade DCIS has central necrosis "comedo" type
Colorectum	Tubular or villous adenoma (adenomatous polyp) with mild, moderate or severe dysplasia	Extent of dysplasia determines the likelihood of conversion to colorectal cancer
Endometrium	Endometrial intraepithelial neoplasia (EIN)	Crowded glands with stroma comprising less than 50% of area.
Esophagus	Focal low and high grade dysplasia in Barrett's metaplasia	Grade of dysplasia is determined by extent of dysplasia
Lung	Bronchoalveolar carcinoma (BAC) is now classified as a pre-invasive neoplastic lesion leading to adenocarcinoma. Atypical adenomatous hyperplasia (AAH) also is postulated to be a pre-invasive neoplastic lesion of lung.	This designation of BAC is questionable due to the aggressive behavior and extent of some bronchoalveolar lesions
Pancreas	Low (PanIN1) and high (PanIN2 and PanIN3) grade pancreatic intraepithelial neoplasia; Intraductal papillary mucinous neoplasia (IPMN); other cystic lesions	Low grade PanIN features mucinous tall columnar cells with basal nuclei which transition to high grade PanIN with dysplastic nuclei and disorganized orientation of cells. IPMN feature large $>$ 1 cm ducts and cystic-like structures lined with dysplastic cells
Prostate	Low (PIN1) and high (PIN2 and PIN3) grade prostatic intraepithelial neoplasia; proliferative inflammatory atrophy	PIN – Expanded duct with reduced numbers of flattened basal epithelial cells forming a discontinuous layer surrounding dysplastic luminal-type epithelial cells
Small bowel	Tubular, tubulovillous, villous adenomas	Villous adenomas are more likely to progress to malignancy
Stomach	Adenomas (sessile or pedunculated polyp) low grade and high grade dysplasia in flat gastric mucosa	Degrees of dysplasia determine low and high grades

dometrium, cervix, lung, pancreas, salivary glands, esophagus, stomach, small bowel and colorectum. Carcinomas develop in the kidney, adrenal, and liver, but these are not usually referred to as adenocarcinomas. Of the adenocarcinomas, only breast, prostate, pancreas, esophagus, stomach, small bowel, colorectum, lung and endometrium have lesions clearly accepted as intra-epithelial or pre-invasive neoplasia (Table 3).

The major characteristics of the different types of intraepithelial neoplasia which ultimately progress to adenocarcinomas are typically both histopathologic and molecular.

Of these, pre-invasive neoplastic lesions of the breast and prostate are similar.

In the case of DCIS and PIN there is an apparent expansion of the duct/ductolobular unit at the site of the lesion caused by the proliferation of neoplastic cells. These neoplastic cells may develop with the molecular characteristics of either luminal or basal cells (Fig. 3). Another is the presence of a single usually discontinuous layer of basal cells and/or myoepithelial cells between the proliferating luminal-like cells and a basement membrane (Fig. 3); stem cells are likely present in this basal cell population. Thus, increased proliferation is a major factor which affects the development of pre-invasive neoplastic lesions. Another feature is the accumulation of molecular changes which have the potential to drive proliferation and/or inhibit apoptosis. Of note, it is the histopathologic lesion that is currently accepted as defining these pre-invasive lesions.

12. Ductal carcinoma in situ and lobular carcinoma in situ as pre-invasive neoplastic lesions of the breast

A range of proliferative changes can be noted in breast tissue, but even the mild proliferative changes noted in fibrocystic changes carry an increased risk for the development of cancer $\approx$ X 1.5. Nevertheless, the separation of atypical ductal hyperplasia (ADH) (a relatively benign change that does not require therapy and has a relative risk of breast cancer 4.4) from low grade DCIS requiring surgical resection with a relative risk 9–11 is the line of separation between benign and a classification of pre-invasive neoplasia [58–60]. The molecular separation of such lesions varies. In general, more aggressive molecular changes are reported to occur less frequently in ADH and in low grade DCIS; also, some cases of atypical hyperplasia with molecular changes have developed as DCIS or invasive cancer has "spread" to uninvolved areas of a duct (Fig. 4).

Ductal or lobular carcinomas in situ of the breast have been identified by their atypical histologic features, their frequent association with mammary carcinoma, and the development of mammary carcinoma after initial surgery for 10 to 15% of cases with DCIS or LCIS. In both DCIS and LCIS, myoepithelial cells are present in an incomplete layer at the base of the lesions (Figs 3 and 4) and whole or partial lobules are sometimes involved by pre-invasive neoplasia

Table 4
Biomarker expression in benign, pre-invasive, neoplastic (PIN) and matching malignant (invasive adenocarcinoma) prostate epithelial cells [2]

Biomarkers		Benign-normal		PIN		Malignant
		Basal	Luminal	Basal	Luminal	
Growth Factors	TGFα	+/-	+/-	+	+	++
Growth Factor Receptors	EGFR; P185erbB-2; P180erbB-3	++; ++; ++	+; +; +	++; ++; ++	+; ++; ++	+; ++; ++
Glycosylated Tumor Antigens	TAG72; Lewis Y	-; ++	-; +	-; ++	++; ++	++; ++
Tumor Suppressor Gene	p53	-	-	-	+/-	++
Anti-metastasis Gene	nm23-H1	++	+	++	++	++
Metabolic Enzymes	FASE	+/-	+	+/-	++	++

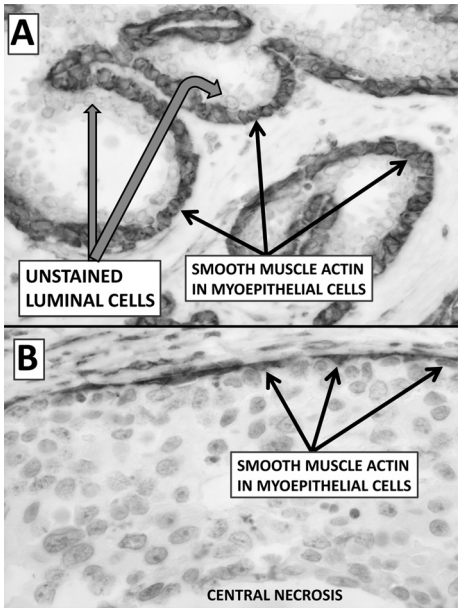

Fig. 3. Panel A (X600) is an immunohistochemical stain of uninvolved ducts (normal appearing ducts in a patient with ductal cancer) of the breast. Note the myoepithelial cells (black arrows) are arranged in a single basal layer, are flattened to cuboidal and stain strongly with smooth muscle actin (SMA). Panel B (X600) is an area of high grade (comedo) DCIS. Note the myoepithelial cells (black arrows) which stain strongly with SMA are flattened or pyramidal, and do not form a continuous layer as they do in uninvolved ducts. The luminal-like cells of DCIS do not stain with SMA and form a cellular compartment which has many layers of proliferating cells.

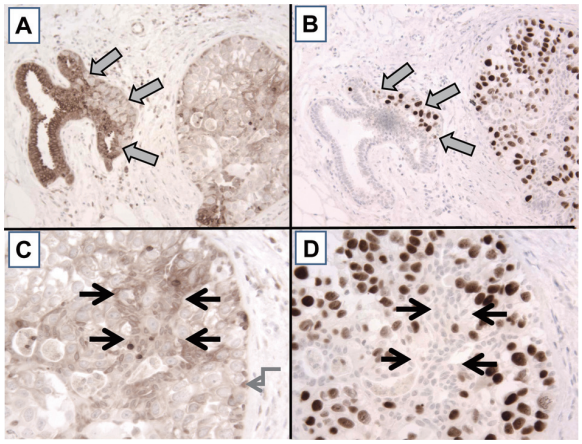

Fig. 4. Depicts an area which resembles atypical hyperplasia to low grade DCIS but this photograph probably represents a tumor invading a duct; also there is an adjacent duct, most of which is normal. Panel A (X200) is immunostained for Bcl-2 and both basal and luminal cells of the uninvolved duct stain strongly with Bcl-2. Note that the architecture of a portion of this duct next to the lesion is disrupted by larger cells staining weakly for Bcl-2 (marked by wide gray arrows). Also, the basal-like cells of the area of duct expansion stain moderately to strongly with Bcl-2. Panel B (X200) is immunostained for p53. It demonstrates that the area of disruption of the uninvolved duct contains cells with nuclear accumulation of p53 (i.e., with a p53 mutation or dysregulation). Panels C and D (X600) show the area of duct expansion from Panels A and B. Note the 4 black arrows demark basal-like cells surrounded by larger cells more typical of the cells of ADH/DCIS. These basal cells stain moderately for Bcl-2 (Panel C) and do not demonstrate nuclear accumulation of p53 (Panel D). The mixed cellularity of this lesion suggests a tumor invading a duct. Note the aggressive cells are either spreading to an adjacent uninvolved duct or are inducing changes in the cells of this uninvolved duct.

(e.g., cancerization of lobules) (Fig. 5). However, in the setting of adjacent carcinoma, there is no reliable method of separating DCIS from cases of carcinoma that have invaded a duct. Low grade DCIS and ADH are difficult to separate even when analyzed by experts. DCIS frequently is defined as involving more than one duct and the lesion has a more uniform population of luminal-like cells. If one considers only pure lesions without a component of invasion, both ADH and low grade DCIS tend to be basal cell negative (CK5/6) as well as negative for HER2 and p53. In contrast, high grade DCIS is frequently positive for HER2 and p53, but negative for ER, PR and Bcl-2. For example, high grade (e.g., comedo) DCIS of the breast may exhibit a strong membrane expression of p185^{erbB-2} and it may have intranuclear accumulation of p53, consistent with a mutation in p53. Of interest, DCIS lesions that have more aggressive molecular changes are frequently associated with ductal carcinomas that have these same changes.

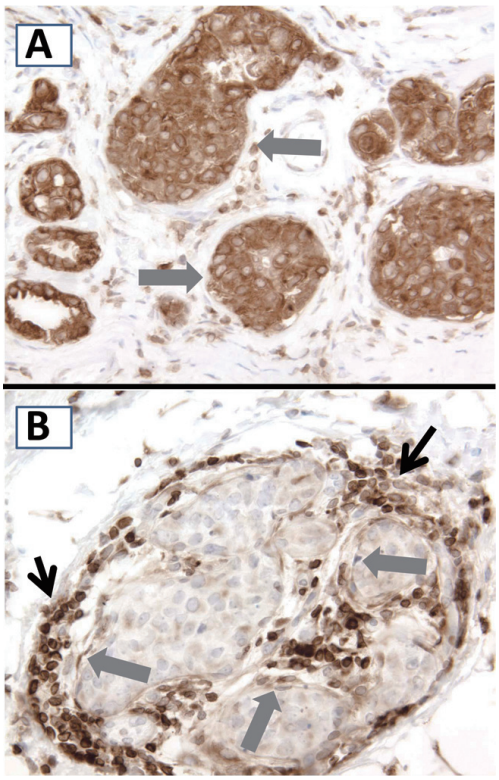

Fig. 5. (X400 immunostained Bcl-2) demonstrates partial cancerization of a lobule; of interest, the cells associated with the cancerization are stained strongly with Bcl-2 (gray arrows). Panel B (X400, immunostained Bcl-2) demonstrates a small lobular area with cancerization of the ductolobular unit. Note lymphocytes surrounding the ductolobular units are staining strongly with Bcl-2 as would be expected; however the remaining basal cells staining with Bcl-2 (gray arrows) are reduced to only a partial layer and there is only weak staining of the luminal cells.

13. Pre-invasive neoplastic lesions of the prostate

The primary lesion recognized as a pre-invasive neoplastic lesion of the prostate is high grade prostatic intraepithelial neoplasia (PIN 2 plus PIN 3). As discussed, with many forms of pre-invasive neoplasia, the lesions are classified as pre-invasive neoplasia because they are associated with cancer [5–7,11]. Also, some pathologists have reported that when there is high grade PIN, especially the cribriform pattern of PIN3, there frequently are microinvasive foci associated with the PIN [61].

When PIN lesions are studied by computerized cytomorphometry, the morphologic features were found to be intermediate between benign normal appearing prostate glands and prostate cancer. These morphometric features included nuclear size (increased), nucleolar size (increased), as well as nuclear size variability and nuclear crowding. Also nucleolar variability was increased – number, size and nucleolar eccentricity. There have been numerous studies of the molecular features of PIN and most show that the molecular features of PIN are similar to the same as the molecular features of prostate cancer [2]. Some investigators have focused on how the molecular features of PIN mirror basal cell markers of normal glands of the prostate [2], while others have emphasized how the molecular features are similar to the luminal cells of normal glands of the prostate. Some of our results are demonstrated in Table 4.

14. Preinvasive neoplasia of the colon

Much of our understanding of the development of colorectal neoplasia (CRN) has originated from the study of familial forms of colorectal neoplasia. The two main conditions of inheritable colorectal cancer are familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC).

As demonstrated in Fig. 1, in the typical development of cancer in FAP, one copy of a mutated APC gene is inherited. This heterozygous state for the mutated APC gene results in increased proliferation of colorectal epithelial cells with the mutated APC gene as well as an increased mutational rate in these cells; thus, colorectal epithelial stem cells would be at risk for developing mutations in the other wild type copy of the APC gene. When cells develop mutations in the second native APC gene, interactions of APC with other molecular pathways change (e.g., β catenin accumulates in the cytoplasm and transfers to the nucleus), proliferation and the mutational rates increase further causing stem cells with homozygous mutations in APC to take over a local area of the colorectum. This causes an architectural change with the formation of atypical crypt foci (ACF) as well as the accumulation of additional mutations including mutations in K-ras and SMAD4 and increased expression of COX-2 (Fig. 1). The architectural disruption continues through the atypical crypt state. At some point, an adenomatous polyp forms and begins to grow. Currently, the “transitional lesion” that leads to adenomatous polyps has not been identified and the development of adenomatous polyps from ACF is not understood. Nevertheless, adenomatous polyps develop. Most small polyps are inflammatory polyps; however, these may develop into serrated adenomatous polyps. As adenomatous polyps increase in size to greater than 3 cm, mutations in p53 become more likely and such

a mutation may be the forerunner of local invasion. These changes are demonstrated in Fig. 1.

The development of many cases of hereditary nonpolyposis colorectal cancer (HNPCC) is similar to adenomatous polyposis coli except the mutated gene that is inherited is frequently one of the DNA microsatellite mismatch repair genes such as MSH2. In HNPCC, ACFs may not develop or be as prominent as they are in FAP or in chemical induction of CRN. Also, as microsatellite instability develops (mutator phenotype), key genes such as TGFβR2 may be inactivated as well as β catenin, bax and caspase 5. The development of HNPCC tumors may be similar molecularly to FAP tumors; however, sometimes inactivation of expression of mismatch repair genes by methylation of their promoters may be involved.

In some cases of HNPCC, neither mutation of mismatch repair genes nor methylation of the promoters of these genes can be identified. There are cases of HNPCC probably secondary to mutations and other changes in multiple genes such as myh which may be occasionally mutated in HNPCC. Alternatively, these cases may be secondary to the inheritance of genes whose polymorphisms may increase the risk of developing cancer. For example, a potential risk factor for the development of HNPCC has been hypothesized to be related to deficiency or haplodeficiency of transforming growth factor β receptor 1 (TGFβR1). The under expression of TGFβR1 has been associated with germline allel-specific expression of several polymorphisms of this gene [43,44].

If an adenomatous polyp of the colorectum is an attached component of an adenocarcinoma, molecular changes noted in the polyp (e.g. mutation in p53) usually are present in the cancer [62]. Thus, in general, molecular changes probably precede histopathologic changes (e.g., mutations in p53 of cells of the colorectum may lead to an invasive tumor with the same dysregulation of p53).

The colorectum is not a homogenous organ, in that the proximal colon (from cecum to proximal 2/3 of transverse colon) develops embryologically from the midget and its vascular system is via the anterior mesenteric system while the distal colon (last 1/3 of transverse colon to rectum) and the rectum develop from the hind-gut and the vascular system is from the posterior mesenteric system. Similarly, the molecular features of the normal proximal colon may vary from those of the distal colorectum [63]. Thus, it is not surprising that tumors of the proximal colon may vary as to their molecular features from those of the distal colorectum.

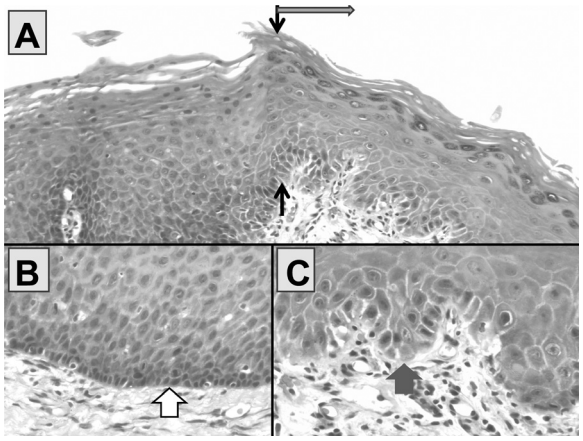

Fig. 6. In Panel A (H&E X200), the black arrows demonstrate the sharp linear transition between an area of mild epithelial hyperplasia of the oral cavity and dysplastic leukoplakia (in direction of gray arrow). A higher power view (X600) of the basal area of the hyperplasia is shown in Panel B and Panel C shows a high power view of the base of the dysplastic leukoplakia. Note that the nuclei are larger in the dysplastic leukoplakia (fat gray arrow) and that the basal area is disorganized and lacks apparent cellular cohesion as compared with the area of mild hyperplasia (fat white arrow). The dysplastic cells demonstrate nuclear accumulation of p53 in about 30% of the basal cells in the area of dysplasia (not shown) and none in area of hyperplasia (not shown).

Also, rectal tumors typically are treated differently than tumors of the colon. Thus, our view is that tumors of the colorectum should not be grouped together in evaluations; instead, for example, tumors of the proximal colon should be evaluated separately.

15. General characteristics of intraepithelial neoplasia leading to squamous cell carcinoma

Squamous cell carcinomas (SCC) develop in the skin, oral cavity, larynx, bronchial system, esophagus, cervix, vagina, labia and anus. For the anus of male homosexuals, cervix, vagina and labia, these lesions are usually associated with a viral infection with one of the human papilloma viruses, usually type 16 or 18 as discussed previously. Also, more controversial is the development of subgroups of oral SCC and bronchial SCCs secondary to HPV infections. Most other squamous lesions usually develop as a consequence of noxious exposures. For the skin, this is UV-B exposure, for the oral cavity and esophagus – tobacco and alcohol, for the larynx and bronchial system – cigarette smoke and/or radon exposure. Such exposures usually cause damage and incorporation of adducts into the DNA of the damaged cells. Subsequently, in the resulting repair

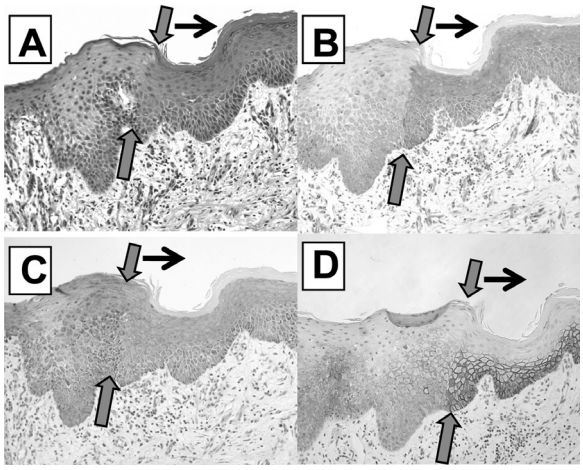

Fig. 7. The panels A-D of Fig. 7 represents a linear transition from mild hyperplasia of the epithelium of the oral cavity to epithelial dysplasia with the separation marked by the gray arrows and the black arrows pointing to the area of dysplasia. Panel A (X200, H&E) represents the histomorphology of this lesion. Panel B (X200, TGFα) demonstrates how increased expression of TGFα follows the exact linear transition of the epithelial boundary as does EGFr in Panel C (X200, EGFr) and CD44v6 in Panel D (X200, CD44v6). Increased proliferation follows this same pattern (data not shown). As will be discussed subsequently, differentiated expression of molecular markers and proliferation follow the boundary of dysplasia almost exactly. The overlap of increased expression of TGFα and EGFr would be characteristic of an area of autocrine interaction.

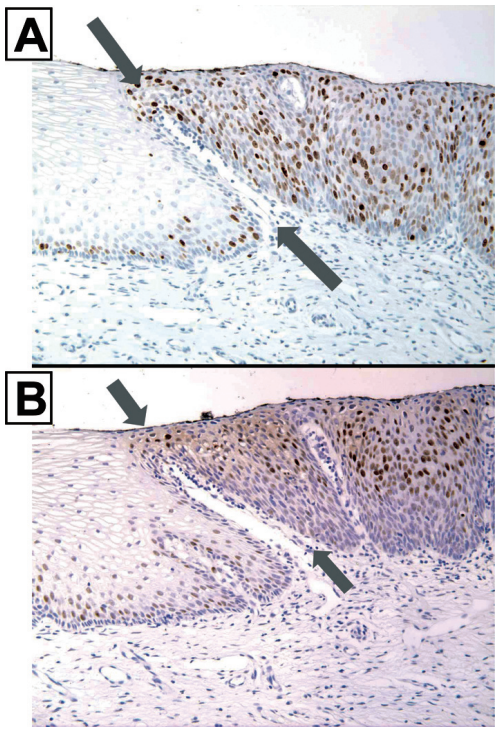

Fig. 8. Represents a transition between normal appearing epithelium of the cervix and CIN epithelium. Black arrows mark the transition. The histomorphometric boundary of CIN is relatively linear and is very distinct. As can be seen, there is increased proliferation in the CIN lesion as measured by Ki67/MIB-1 in Panel A (X200, Ki67/MIB-1) and by PCNA in Panel B (X200, PCNA). Proliferation in the uninvolved (normal appearing) cervix is primarily limited to the base of the lesion, but in CIN, is throughout the lesion. In sections not shown of this same area, increased expression of EGFr and erbB-2 follow the exact histopathologic boundary as does the proliferation and as would be expected, there is not a mutation or dysregulation of p53.

and replication of DNA and the associated proliferation the adducts increase the likelihood that deleterious, somatic mutations may develop. Continuing noxious exposures causing damage and repair are a subtype of LOCDIR lesions.

The pre-invasive neoplastic lesions leading to SCCs are designated as intraepithelial neoplasia primarily in the cervix as previously discussed. In the skin, these pre-invasive neoplastic lesions are designated as actinic keratoses and in most other sites they are referred to as dysplasia/carcinoma in situ. "Dysplasia" in oral lesions is not in general graded because accurate correlations between the histopathology of these lesions and their likelihood of progressing to cancer have not been made as they have for cervical lesions.

Oral dysplasia usually is a lesion with extensive extracellular keratin on its surface (Fig. 6) which gives the lesions a white-gray appearance on visual examination. This appearance of oral dysplasia is called leukoplakia. Other areas of oral dysplasias may have extensive subepithelial vascularity and these lesions are called erythroplakia. Our experience from studies of longitudinal biopsies of leukoplakia is that squamous dysplasia of the oral cavity is a lesion whose boundaries move over a period of several months [64]. Of interest, is that the boundary is not only a boundary in which a profound difference in histopathologic appearance on H&E staining, but also is a boundary of extensive molecular changes. Typically the boundary of an area of squamous dysplasia is quite sharp and linear. This is demonstrated in Fig. 7, Panel A, in which the boundary on H&E staining is easily identified. Also, the boundary of dysplasia is marked by a matching boundary of sharp molecular changes, including TGFα, EGFr and CD44v6 as shown in Panels B, C and D. In other boundaries of dysplasia, both the nuclear expression of p53 and membrane expression of p185^{erbB-2} have been shown to increase in such dysplastic cells of the oral mucosa.

Areas of transition between CIN and uninvolved cervical epithelium and between oral dysplasia and uninvolved epithelium are usually very distinct due to

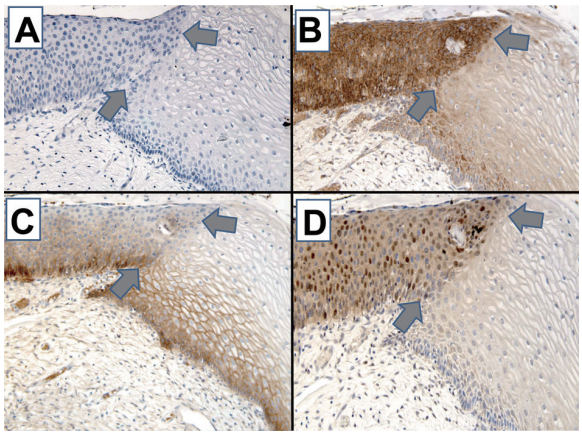

Fig. 9. Represents molecular changes occurring between apparent normal appearing uninvolved epithelium of the cervix and CIN. In Panel A (X200, p53 counterstain hematoxylin), there is no staining for p53 as would be expected. Note how clearly the basal cells are arranged in the uninvolved epithelium and how disorganized they appear in the area of CIN. Like the prior cases, the transition between uninvolved epithelium and the epithelium of CIN is linear (gray arrows point to edge of lesion, and top gray arrow to the direction of lesion). Proliferation (not shown) follows this sharp boundary as does increased expression of erbB-2 in Panel B (X200, erbB-2), EGFr in Panel C (X200, EGFr) and TGFα in Panel D (X200, TGFα). The increased expression of TGFα and EGFr in the CIN lesion may suggest an autocrine interaction within CIN.

the histomorphological changes as shown in Figs 6–9. These areas of transition also demonstrate distinct molecular changes as well as physiologic changes including increased proliferation (Fig. 8). Of interest, the molecular changes of pre-invasive neoplasia usually mirror the molecular changes observed in the tumors which can develop from these pre-invasive lesions. For example, mutations in p53 as represented by visible nuclear accumulation of p53 does not usually occur in SCC of the cervix or in CIN lesions (Panel A, Fig. 9) while visible nuclear accumulation of p53 is common in both SCCs of dysplastic epithelium of the oral cavity as well as in SCCs of the oral cavity.

Of interest, pre-invasive neoplastic lesions of squamous cell type (e.g., CIN or oral dysplasia) do not appear to gradually develop from the basal cells and do not involve the adjacent epithelium as, for example, spreading laterally; rather, there is a sharp boundary that is almost perpendicular to the basal cells. Note the patterns that we do not see as shown in Fig. 10 are informative; we see only pattern (D).

Why is this boundary so sharp? This may be due to a tendency of neoplastic lesions to "stick" together via various attractions such as those described by Norton et al. [65,66].

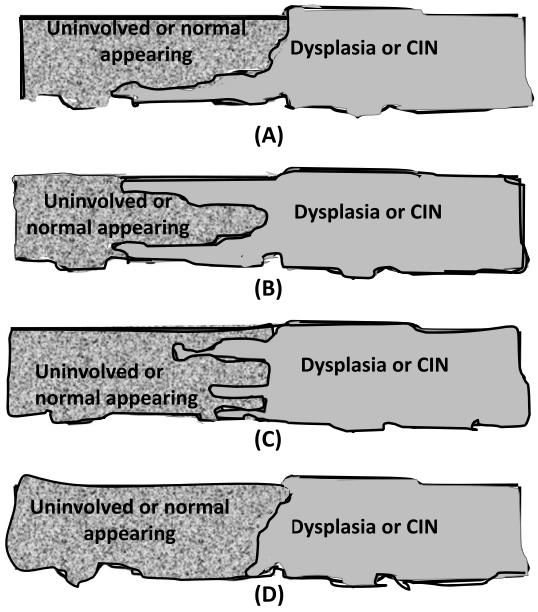

Fig. 10. The 4 cartoons demonstrate 4 potential patterns which could mark the spread of a squamous pre-invasive lesion. In general, the patterns (A), (B), and (C) are not usually noted, but pattern (D) is seen in patients with dysplastic lesions of the oral cavity and/or CIN lesions of the cervix, as shown previously.

15.1. Other pre-invasive lesions

Pre-invasive neoplasia leading to urothelial neoplasia, sarcomas or hematopoietic neoplasia are beyond this section in that they represent a small component of pre-invasive neoplasia or are not currently well defined.

15.2. Treatment of pre-invasive neoplastic lesions

Some better known in situ neoplastic lesions are treated while other in situ lesions are not. The best four examples of organs in which in situ lesions are treated are DCIS and LCIS of the breast and CIN2 – CIN3 of the cervix, actinic keratoses of the skin and leukoplakia of the oral cavity. For example, the whole area of DCIS of the breast is surgically removed and in addition, if complete removal is uncertain, microinvasion is suspected, or the lesions are very high grade, the area of the breast may be subjected to radiation therapy after surgery. Such treatment appears very effective although this is difficult to ascertain because the natural histories of DCIS and LCIS are not known in detail. Nevertheless, in UAB's experience, less than 10% of cases return as either DCIS or invasive cancer. For some CIN2, but all CIN3 of the cervix, the area where CIN typically develops is treated with a laser, cryotherapy and/or is removed surgically.

16. Mouse models of inheritable pre-invasive neoplasia

One of the important aspects of identifying the molecular changes that occur in inheritable cancers is that the pre-invasive neoplastic lesions can be reproduced in some organ systems of transgenic mouse models that carry the same or very similar specific genetic changes that have been identified in inheritable neoplasia in humans. As discussed by Sandgren [67], "Mice are not people, and thus, how do we transfer our understanding of the disease in mice to disease in humans?" He proposes 3 criteria – 1) Morphology – at all levels – gross, microscopic and ultrastructural, i.e., the neoplastic lesions of mice and men should look alike. 2) Molecular – human and murine tumors should display the same molecular alterations and 3) Behavioral – the neoplastic lesions of mice and men should have similar growth, invasiveness, metastases and effects on the species. To this I would add a 4th, Normal Biology – the biology of the cells of the organ of interest should be similar in mouse and man. For example, because the biology of the prostate is different in mice and men (e.g., cellular location of androgen receptors), it is unlikely that excellent mouse models are likely to be developed for the prostate. Thus, transgenic mice do not develop models of useful development of neoplasia for all organ systems usually because the model of disease in rodents does not mirror some aspect of the disease in humans.

16.1. Mouse FAP

A transgenic model of familial adenomatous polyposis (FAP) of the colon is the multiple intestinal neoplasia (MIN) mouse which carries a germ-line mutation in the mouse homologue of the APC gene (Apc^{Min}). Even though this model primarily develops many small adenomas of the small bowel, it has been quite useful in studying pharmacologic agents that may modify intestinal tumor development such as COX-2 inhibitors which markedly inhibit adenoma formation in this animal model. Also, when crossed with a mouse haplodeficient in TGFβR1, large dysplastic adenomas develop in the colon indicating that TGFβR1 may be an important gene in the development of CRN that interacts with Apc^{Min} [44].

16.2. Mouse PanIN

Similarly there are several mouse models of pre-invasive pancreatic cancer. One of the more interesting aspects of the embryologic development of the pancreas is that both the exocrine pancreas and the endocrine pancreas are hypothesized to develop from the cells of the ductal epithelium of the pancreas. This can be observed in some transgenic mice models in which ductal-alveolar metaplasia as well as islet metaplasia can be observed frequently in association with pancreatic ducts [68,69]. Very rarely we and others have observed islet metaplasia in a human pancreatic adenocarcinoma or mixed acinar-endocrine carcinomas [70].

Pre-invasive neoplasia of the pancreas is also a lesion for which a pattern of genetic changes have been identified. Similar to other organ systems, pancreatic intraepithelial neoplasia (PanIN) is designated as PanIN 1, 2 and 3 in which PanIN3 is the most severe lesion and hence the lesion at greatest risk for developing pancreatic cancer. Histopathologic models which mirror the various grades of PanIN as well as the biological changes of PanIN develop in various murine models of pancreatic neoplasia.

The typical ductal adenocarcinoma of the pancreas (PDAC) frequently carries mutations in p16/CDKN2A, p53, SMAD4/DPC4 and K-ras. Because about 90% of PDACs are aneuploid due to early chromosomal instability secondary to defective telomeres, some of these same genes (the suppressor genes, p16, p53 and SMAD4) also are affected by LOH via partial chromosomal loss of 9p, 17p, 18q, respectively. Losses of portions of multiple other chromosomes are also characteristic of pancreatic neoplasia though less common.

Mutations of K-ras-2 begin in PanIN and continue to accumulate in more advanced lesions until approximately over 90% of PDACs have K-ras-2 mutations. Of tumors with native K-ras-2, 1/3 have defects in BRAF downstream of K-ras-2. BRAF is present in about 7% of PDACs, but only in those without mutations in K-ras-2. Of interest, mutations in BRAF occur much more frequently in mismatch repair deficient cases of cancer. Mutations or homozygous deletions of p16 also begin in early stage pancreatic neoplasia until about 80% of tumors are involved. Almost all the other advanced PDACs have hypermethylated p16 or dysregulation of Rb or Cyclin E. In contrast, p53 which is mutated and inactivated in 50–70% of PDACs is a late event in the development of pancreatic neoplasia.

The early mouse models of pancreatic cancer demonstrated several interesting features. In some of the first models, the elastase gene regulatory elements (Ela) which are found specifically in pancreatic acinar cells were combined with one of several molecules associated with pancreatic cancer. These included TGFα which

when induced in transgenic mice produced a severe diffuse pancreatic fibrosis and acinar to ductal metaplasia. When the TGFα transgenic mice were combined with p53 null mutations, exocrine carcinomas developed in all mice within 4 months; these lesions were complex, but did not metastasize. Of interest, 50% of p53 heterozygous crossed animals developed methylation of the promotor of CDKN2A, DNA gains affecting EGFr, Rel and c-myc and DNA losses of Rb pathway. Subsequently, the Ela promoter was used with mutant (activated K-ras) to produce mice, but activating acinar to ductal metaplasia and PanIN and IPMN lesions. When K-ras mutations were combined with p53 null mice, acinar carcinomas that could metastasize developed [67].

Subsequently, mice with activating K-ras mutations were combined with mice with deletions of Ink4a/Arf (p16/p19) tumor suppressor alleles; this produced early development of PanIN lesions which rapidly developed into highly invasive and a metastatic pancreatic cancers that caused deaths of all such animals by 11 weeks; however, SMAD4 protein was not lost and p53 was native. Also, EGFr and HER2/neu were overexpressed [68].

A recent study of mice with activating K-ras mutations and loss of SMAD4 found that these mice developed PanIN and IPMN-like lesions as well as locally invasive pancreatic exocrine lesions. Ductal-alveolar metaplasia also was noted. Thus, many of the molecular changes observed in pancreatic ductal adenocarcinoma and the pre-invasive lesions of this tumor (PanIN and IPMN) are features of mouse models of pancreatic cancer [69–72].

17. Summary

In summary, there are multiple causes of pre-invasive neoplasia ranging from radiation, chemicals, infections, and long-term continuing damage, inflammation and repair. Pre-invasive neoplasia may progress to invasion, may remain static for many years or may regress. Some types such as DCIS/LCIS, oral dysplasia, adenomatous polyps and cervical dysplasia are well described and are treated. Other forms such as high grade PIN and PanIN remain as warning signs of an at risk state.

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Translational pathology of neoplasia

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Abstract. With the increasing use of individualized medical care (personalized medicine) in treating and managing patients with cancer, the utilization of biomarkers in selecting and tailoring such medical approaches also is increasing and becoming more important. Specifically, many therapies are effective against only a subgroup of a specific type of tumors and exposing patients with different non-responsive subgroups of the same tumor to ineffective therapies, not only exposes these patients needlessly to acute and chronic side effects of the therapy, but also adds to the costs of medical care. For example, the Oncotype Dx test for estrogen receptor positive tumors that are node negative has been used to identify low risk tumors for which surgery alone is an adequate therapy. Biomarkers may be used to aid in multiple aspects of medical care related to cancer, including early detection, diagnosis, risk assessment, as well as in predicting the aggressiveness of cancers (i.e., prognosis) and predicting the therapeutic efficacy of treatments (i.e., prediction). Biomarkers may be also used as surrogate endpoints to aid in evaluating therapies and preventive approaches. Types of biomarkers vary greatly and include histopathologic appearance, stage of the lesion, quantitative morphologic features, size of the lesion, metastatic pattern and extent of metastasis, as well as imaging and molecular features. The types of measurements of biomarkers also vary; for example, molecular features can be measured at the DNA, mRNA or protein levels as well as at regulatory levels (e.g., microRNA). The usefulness of each biomarker is limited by its sensitivity and specificity in fulfilling its role (e.g., in early detection) and the requirements of sensitivity and specificity to accomplish specific tasks are affected by multiple variables. For example, both very high specificity and sensitivity of a test are required to screen a population with a low prevalence of a specific tumor. The goal of this manuscript is to introduce the reader to how biomarkers may be used and the limitations on the uses of biomarkers in translational research.

Keywords: Sensitivity, specificity, early detection, prognosis, risk assessment, surrogate endpoints, diagnosis, receiver operating characteristic, prediction, biomarkers, prevalence, medical costs, side effects, histopathology, molecular features, imaging, prevention, treatment, personalized medicine, individualized medical care

1. Introduction

Over the last 50 years of basic research, numerous genes, proteins, signal transduction pathways and other molecules (e.g., microRNA) have been identified that are differentially present in pre-invasive neoplasia and in cancers compared to normal tissues. In addition to the identification of the genes of the human genome, literally thousands of other modified proteins (e.g., phosphorylated or splice variants) also have been identified whose phenotypic expressions are modified in neoplasia and similar processes such as tissue repair or inflammation.

Translational research is research in which any of these molecules or molecular pathways are translated into being useful clinically, i.e., in practical medical uses that directly affect medical care. Such uses include aids in early detection, determination of clinical outcomes (prognosis), diagnosis, detection of recurrence after therapy, risk assessment, identification of targets for therapy, prediction of responses to therapies (i.e., prediction), monitoring clinical outcomes of therapies

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(i.e., surrogate endpoints), and imaging diseases processes [1–31]. These areas of translational research are discussed subsequently.

1.1. Early detection

It is very important to identify disease processes as rapidly as possible in order to limit the damage of the disease, to treat the disease at a stage at which the disease can be more easily managed and/or to diagnose the disease when it can be cured. The easiest stage to cure a neoplastic disease is at the stage of pre-invasive neoplasia [32]. If all and subsequent recurrent pre-invasive neoplasia (PINN) can be eliminated from a patient, then PINN will not become invasive and hence the neoplastic process will not become life threatening. For example, with the use of the PAP smear, pre-invasive squamous neoplastic lesions of the cervix (cervical intraepithelial neoplasia [CINs]) can be identified and removed or treated before the lesion invades; thus squamous cell carcinoma (SCC) of the cervix can be prevented in a society which screens and treats all women for high grade CIN. Because of the nature of some forms of PINN, the pre-invasive lesions may recur following successful therapy; however, the time course of the disease usually does not increase so the potential development of invasive disease would be delayed after each removal of PINN.

The goal of the translational pathology of PINN is the reliable identification of the lesion by the least invasive and most accurate methods possible. For example, in screening for CIN, originally the screening test was principally by histopathological examination of the cells removed during the scraping of the squamous columnar junction of the cervix. If CIN2 or CIN3 were detected by cytologic examination, then a colposcopic examination and biopsies of cervical lesions would dictate whether or not there was a need for further treatment or for careful follow-up. With the understanding of the biology of CIN, the over treatment of some CIN2 which might not progress and the viral origin of CIN, the medical evaluation of CIN now may include a measure of the types of human papilloma viruses (HPV) present in the lesion [32]. If strongly pathogenic HPVs are detected (e.g., HPV16, 18, 31, 33 or 45), the likelihood of the progression of CIN to SCC is greatly increased. HPV analysis can now identify 13 high risk types of HPV; an extensive study of HPV screening in India indicated that HPV screening identified more cases of cancer and reduced cancer deaths compared to cytologic screening [33]. Also, the understanding of the etiology of cervical cancer has led to vaccine therapy directed at specific pathologic strains of HPV, e.g., HPV 16 and 18 [34].

The screening for neoplastic processes other than cervix has followed different approaches. For example, radiological imaging of the breast by mammography has led to the detection of small lesions in the breast and hence the early identification of not only invasive mammary carcinoma, but also in situ carcinoma. Even with the success of the early detection of cervical cancer and breast cancer in the industrially developed world, because of the costs of these methods of early detection, such screening has not been widely adopted in developing countries.

A major goal of early detection is a test of high sensitivity and specificity that can be performed on easily obtained bodily fluids/samples such as blood, urine, saliva, or feces. A major feature of an early detection test is its sensitivity and specificity. The sensitivity of the test should permit most cases of the neoplastic process to be identified while it is curable. Thus, an early detection test is of little use if it only can identify a large tumor burden which is primarily correlated with large invasive tumors or metastatic disease. For example, the soluble component erbB-2 (HER2/neu) or $p105^{erbB-2}$ was found elevated primarily in more advanced cancers of the prostate [35] and breast. Also of great concern is the specificity of a test because the rate of false positives which are inversely related to the incidence of the neoplastic process, are very important, especially when the next step after the positive results is of great expense or morbidity.

Some of the major serum/plasma tests for diseases are listed in Table 1. Each of these tests has problems associated with its use as "screening tests" for the early detection of neoplasia [36]. For example, prostatic specific antigen (PSA) is produced by normal prostatic glandular cells and hence benign prostatic hyperplasia (BPH) also causes an elevation of PSA [37–39]. This especially is a problem because BPH causes elevations of PSA that are in the range 2.5–10 ng/ml – the early abnormal range of PSA in which the PSA marker would be most useful in early detection of prostatic cancer. Nevertheless, the use of PSA as a screening test has been adopted because the diagnosis of prostate cancer is so frequent in men over 50 years of age; as a consequence of an elevated PSA, a 12 core biopsy of the prostate is likely to identify prostate cancer. In contrast, the CA125 test is a relatively sensitive test for ovarian carcinoma; yet CA125 is not frequently used as a screening test for ovarian cancer in an asymptomatic,

Table 1
Serum/plasma biomarkers in neoplasia

Serum/plasma marker	Source malignant lesion
PSA	Prostatic adenocarcinoma
CA125 (MUC 16)	Female – ovarian, epithelial cancer; GI cancers; endometrial cancers
CA19.9	Pancreatic carcinoma
CA15-3, CA27.29 (MUC 1)	Prognosis of breast cancer; response to RX of breast cancer
CEA, CEACAM-1	Gastrointestinal carcinoma, pancreatic carcinoma
AFP	Hepatocellular carcinoma, germ cell, nonseminoma
hCG	Germ cell – seminomas; choriocarcinoma
$p105^{erbB-2}$	Breast, prostate, ovarian cancers
Long DNA	Many types of cancer, e.g., in feces, colorectal cancer
TPA*	Many types of cancers
Circulating tumor cells (CTC)	All cases of cancer

*Tissue polypeptide antigen (TPA) is a heterogenous combination of cytokeratins 8, 18, and 19 that can be measured in serum or urine; TPA has been considered as markers of cellular proliferation or apoptosis and hence maybe increased in patients with tumors [40,41].

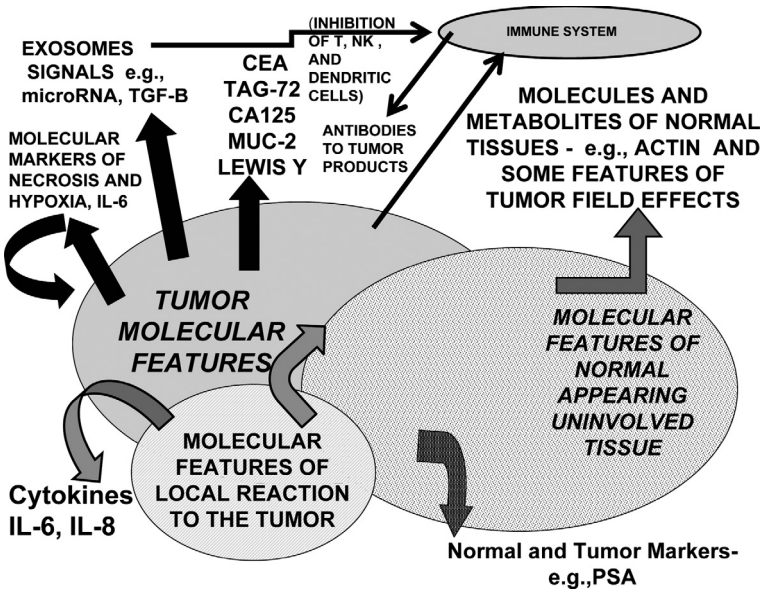

Fig. 1. Demonstrates the complex interaction between a tumor, its surround and the immune system. It shows how various biomarkers may be associated with the presence of a tumor without coming directly or uniquely from a tumor. For example, PSA is produced at higher levels in the cells of normal glandular epithelia of the prostate than in cells of prostate cancer.

low risk population of women because of its specificity and the high rate of false positives that result because ovarian carcinoma is relatively uncommon [36].

The above issue is demonstrated as follows: If a test is 100% sensitive and 90% specific, but a tumor occurs in only one in 1000 patients, in testing 1000 patients the test will identify the one patient with tumor (true positive e.g., $\frac{\text{true positives (TP)}}{\text{false negatives + TP}} = 1.0$), but also over 10% or 111 patients without tumor (false positives e.g., $\frac{\text{true negatives (TN)}}{\text{false positives + TN}} = 0.9$). In this case the issue on using the test in screening becomes the cost and morbidity associated with follow-up of each of the 111 false positive cases with a more specific test. Thus, the cost, morbidity of follow-up testing, and acceptability of testing as a screening test should be balanced with decreased morbidity and mortality of the disease based on using the screening test [36].

Another limitation of many biomarkers for the early detection of a specific neoplastic process is that many biomarkers are not specific for one type of tumor. For example, increases in serum levels of interleukin 6 (il-6), il-8 or CEA may occur in patients with many types of cancer (e.g., pancreatic or colorectal cancers) as well as some inflammatory processes. Thus an elevated level of a biomarker may indicate a potential tumor in

multiple sites (e.g., lung and colon). This may be more likely when the elevation of the screening biomarker is secondary to a general immunological or tissue reaction to a tumor, rather than to a product released specifically by a tumor (38, Fig. 1). For example, acute phase reactants may be elevated secondary to the body's reaction to a tumor rather than by being produced specifically by the tumor.

The challenge of early detection is to find a marker(s) that are both sensitive and specific and that can be detected, based on biomarker levels in bodily fluids or image/fine needle aspiration, when a neoplastic process is curable (i.e., when the neoplastic process is small or pre-invasive). Except for prostatic specific antigen, most biomarkers that have been reported are not tumor or organ specific. Because a single marker that is both sensitive and specific as well as organ specific has proved to be very difficult to identify for most organ systems or specific tumors, a combination of biomarkers may need to be used in early detection of specific tumors. In this combination of biomarkers, some biomarkers might add sensitivity and others specificity.

1.2. Detection of recurrence or metastatic disease

While not very useful in screening for initial disease, most of the molecules listed in Table 1 are very useful to detect the recurrence of tumors which have been surgically removed or treated with radiation. For example, when the prostate or the source of PSA has been removed either by surgery, successful radiation or hormonal therapy, all of which act on normal as well as neoplastic prostate tissue, PSA should drop to the minimum detectable range; thus, a subsequent consistent increase in PSA is indicative of recurrence or of metastatic disease. Similarly, a continued elevation of CA125 following removal of both ovaries or a subsequent rapid increase in CA125 is usually indicative of recurrence, metastatic ovarian carcinoma or rarely a different primary cancer, that may also produce CA125, for example, pancreatic cancer.

1.3. Diagnosis

The use of biomarkers in diagnosis is closely related to the use of biomarkers in early detection and/or to detect recurrence. The only difference between early detection and diagnosis may be the stage of the disease being detected or diagnosed and the accuracy of the detection. Thus, a method that is 100% sensitive and 100% specific for the early detection of a neoplastic process is actually a diagnostic method. The actual diagnosis of neoplastic lesions may be based upon measurement of biomarkers in either the tumor tissue or in bodily fluids. For example, biomarkers may be very useful in aiding the diagnosis of neoplasia when they are specifically expressed in the tissue of the neoplastic process. Depending upon the biomarker, such biomarkers may be identified by histochemical assays, immunological assays or by microchemical methods.

Over the last three decades, the use of immunohistochemistry to identify biomarkers useful in the diagnosis of neoplastic processes has revolutionized the practice of pathology with a major shift from subtyping tumors using histochemistry to using immunohistochemistry which is usually more specific and more sensitive than histochemistry. For example, melanomas were once diagnosed by using the Fontana-Masson histochemical stain which identifies by staining with a silver solution cells with Argentaffin characteristics. In contrast, currently many immunohistochemical stains which are more specific for melanomas such as MELAN A, HMB45 or S100 are used to diagnose melanomas.

Some biomarkers such as number of mitoses have also been used to separate malignant (e.g., leiomyosarcomas) from benign tumors (e.g., leiomyomas). Such a pathological separation is beyond a marker of prognosis which usually applies to separation of more aggressive from less aggressive cancers as will be discussed subsequently. Several immunohistochemical markers can substitute for counting mitoses (Ki67, PCNA) or may aid in counting mitoses.

In some cases with limited material (small biopsies with a few foci of atypical cells), biomarkers may be used as an aid to separate malignant from benign lesions. For example, in prostate and breast, the presence of basal cells may separate malignant from benign glands because malignant glands typically do not have both luminal and basal cells. In breast, S100 and smooth muscle actin may be used to identify basal myoepithelial cells. In the case of the prostate, basal cells are stained with a basal cytokeratin (34βE12) or p63. Similarly, new markers such as α-methylacyl-CoA-racemase (AMACR) may be expressed in a proportion of malignant cells of the prostate [42] but not in normal or uninvolved prostate cells.

Recently, biomarkers have been increasingly used to subdivide or subtype tumors such as breast carcinomas. By the original classification of breast carcinomas by histopathological analysis several main subtypes were characterized including ductal, lobular,

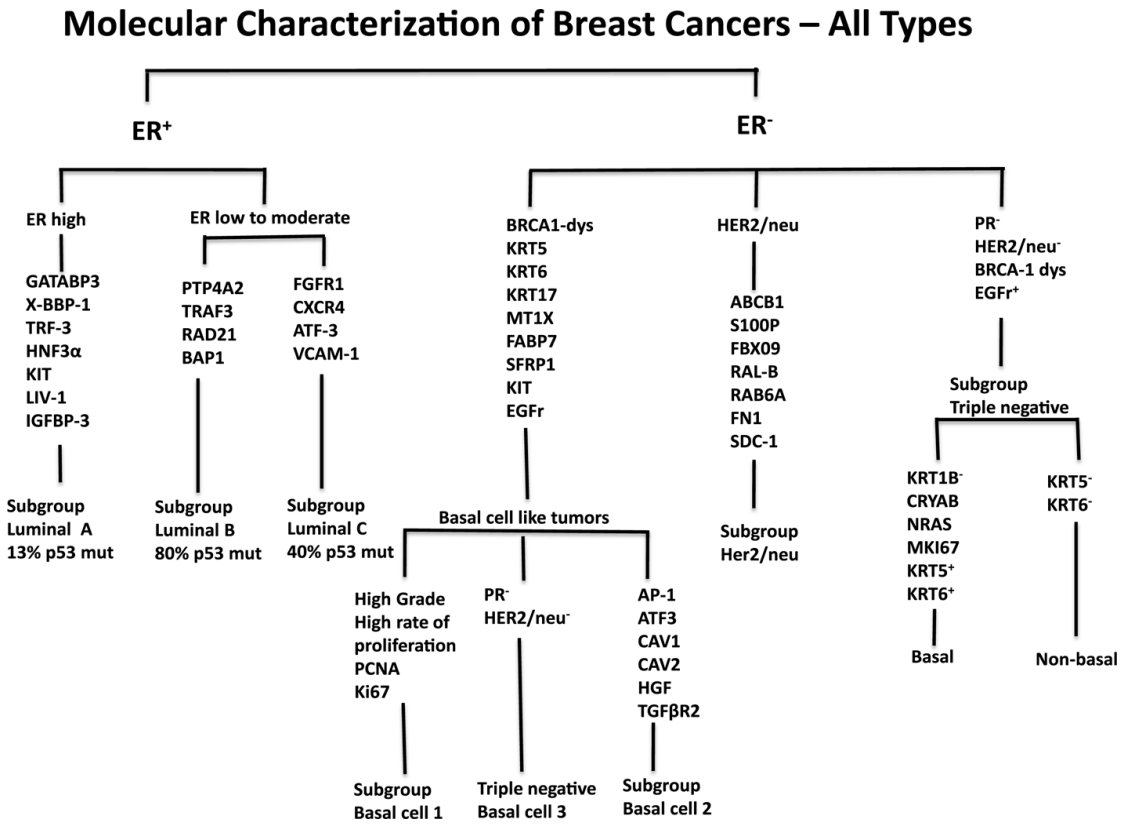

Fig. 2. Demonstrates the molecular subtyping of breast carcinoma. The abbreviations used are defined subsequently. (GATABP-3) GATA binding protein 3; (X-BBP-1) box binding protein 1; (TRF-3) trefoil factor 3; (HNF3α) hepatocyte nuclear factor – 3α; (PTP4A2) protein tyrosine phosphatase type IVA member; (TRAF3) Tumor necrosis factor receptor associated factor 3; (BAP-1) BRCA associated protein 1; (KPT5, -6 or -17) keratin 5, 6 or 17; (MTIX) metaiothionein 1x; (FABP7) fatty acid binding protein 7; (SFRP1) frizzled related protein 1; (ATF3) activating transcription factor 3; (CAV1 or 2) caveolin 1 and 2; (HGF) hepatocyte growth factor; (TGFβR2) transforming growth factor β receptor II; (ABCB1) multidrug resistance protein 1; (S100P) S100 calcium binding protein P; (FBX09) fatty acid syntase; (RALB) GTP binding protein; (RAB6A) member of RAS oncogene family; (FN1) fibronectin 1; (SDC1) syndecan 1. In the future, the complex cancer such as lung or renal carcinomas also are likely to be subdivided molecularly.

medullary, tubular and papillary. The molecular analysis of breast cancer has further subdivided the above lesions based on molecular expression [43–50]. The luminal type of breast cancer has been characterized primarily by ER^+ cells and this group was subdivided into Luminal Type A, B and C. In contrast, breast cancer with ER^- cells has been subclassified as of basal type (1 and 2), HER2/neu (erbB-2) subtype, or a triple negative subtype. Of interest, there is significant overlap between basal type (BT) and triple negative breast cancers (TN) with perhaps 28% of TN as being non-basal subtype and 23% of the basal type being non-TN. There also is a strong overlap of both groups with some type of dysregulation in BRCA-1 [48–50]. See Fig. 2. Of interest only medullary carcinoma is associated with primarily one molecular phenotype, the basal cell subtype.

Another use of biomarkers in diagnosis is the identification by biomarker expression of the sources of metastases when a primary cancer has not been identified. In some cases, the marker, such as prostatic specific antigen (PSA), is very accurate in identifying metastatic foci of prostate cancers in tissues. In other cases, the demographics of the patient, the pattern of metastases (e.g., in bone) plus the expression of biomarker is useful. For example, an undifferentiated epithelial-like cancer in the axillary lymph nodes of a woman is a melanoma if the cancer expresses MELAN A, but is likely a breast cancer if the lesion does not express MELAN A, HMB45, or S100, but instead expresses generalized mixtures of high and low weight keratins such as AE1/AE3. Alternatively, the lesion may be a lymphoma if it expresses CD3 or CD20. A general molecular approach to characterizing a metastatic lesion is depicted in Fig. 3.

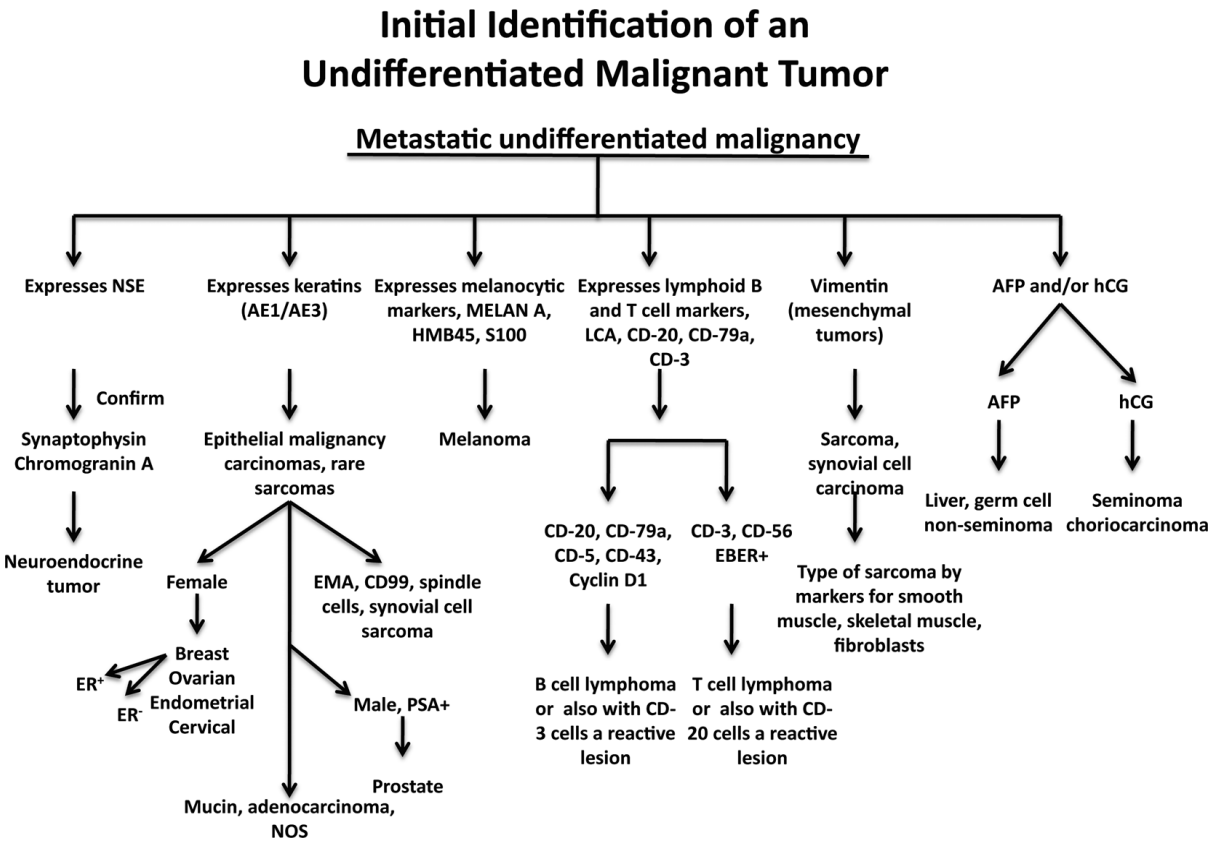

Fig. 3. Demonstrates how molecular analysis aids in identifying poorly differentiated malignant tumors, especially metastatic cancers, whose primary cancer is unknown. Typically the pathologist may suspect which type of tumor is the primary cancer and only a few of these molecular markers are used to confirm the initial impression.

Thus, to determine the source of a metastatic lesion, the pathologist and oncologist must rely on the history of the patient as to prior malignant lesions. If none, then the demographics of the patient and the pattern of metastases are important in identifying the primary lesion. For example, metastases only to local lymph nodes and especially to a sentinel node is usually indicative of the location of a lesion draining to these nodes. Finally, each type of cancer has a typical pattern of metastatic spread. For example, gastrointestinal carcinomas metastasize to local lymph nodes and then to the liver, while prostate cancer metastasizes to local lymph nodes and then to bones, especially the vertebral column. After the clues provided by the sex and age of the patient and the pattern of metastases, the pathologist and oncologist frequently must rely on molecular markers to confirm the primary tumor.

Biochemical markers in serum/plasma can also be used in diagnosis of neoplasia. For example, small adrenal cortical tumors producing primarily cortisol and the clinical picture of pure Cushing's syndrome are likely benign; however, an adrenal cortical tumor producing excessive androgens is likely malignant. Similarly, a small paraganglioma of the adrenal gland producing primarily norepinephrine is more likely to be benign than a similar paraganglioma of the same site producing primarily dopamine; paragangliomas at other sites (e.g., paravertebral) may have a greater rate of malignancy. Also, the identification of a paraprotein of 10 g/L in serum may be indicative of a plasmacytoma or multiple myeloma, but a paraprotein of $<$ 10 g/L may be indicative of a process that may not progress to a plasmacytic malignancy or to a lymphoma.

1.4. Surrogate endpoint biomarkers

Evaluation of some clinical endpoints such as the prevention of malignancy by a chemopreventive agent may take several years and involve thousands of patients in order to complete the analysis. Thus, such testing can be prohibitively expensive when many agents must be evaluated. If a biomarker(s) could be identi-

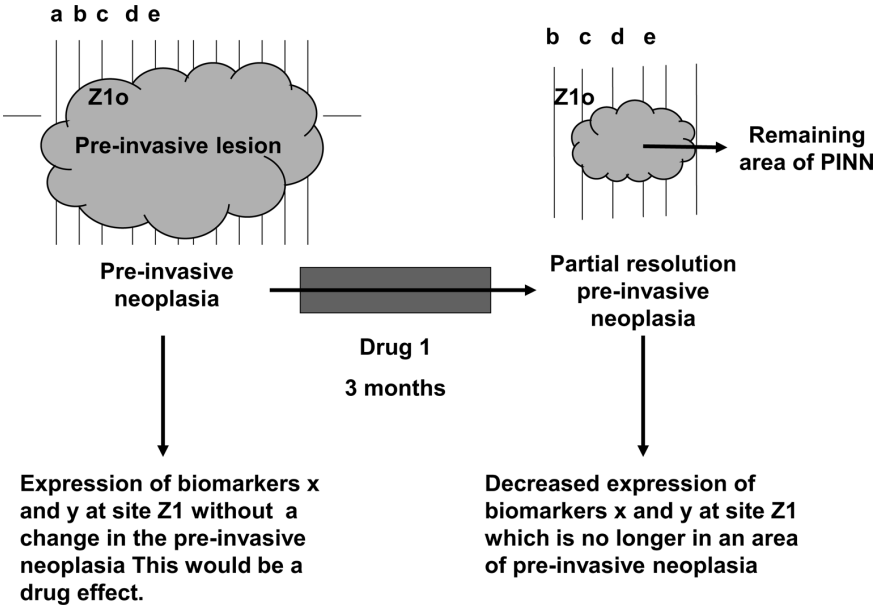

Fig. 4. In Fig. 4, note biomarkers x and y may change at site Z1 without the resolution of the area of pre-invasive neoplasia. This would correlate with an effect of the drug and not a pattern indicative of a surrogate endpoint. Similarly, areas of pre-invasive neoplasia may "move" spontaneously, with or without resolution of the extent of the PINN. In this case, there is partial resolution of the area of PINN. The movements of PINN lesions over a period of months without even partial resolution of the PINN is why the use of "controls" is so important in studies to identify responses in SEBs to drugs used in the prevention of neoplasia [51].

fied for which changes in the biomarker(s) correlated exactly with a preventive response of an agent, then changes in such biomarker(s) could be used as surrogates to the clinical response; such markers are called surrogate endpoint biomarkers (SEBs). If a biomarker always changed many months to years before a preventive response, then the biomarker could be used as a SEB, i.e., as an inexpensive screening method to determine the effectiveness of preventive agents; then if SEBs indicated that a preventive agent was likely to be successful, the preventive agent could be evaluated by treating a population in which changes in SEBs could be correlated with partial or complete resolution of PINN. Thus, exhaustive clinical trials could be undertaken only for those agents that were most promising in initial trials. It is important to understand that the SEBs should correlate with clinical prevention of the lesion (e.g., complete or partial resolution of a preinvasive neoplastic lesion) and not be only correlated with administration of the clinical drug. Consider Fig. 4.

An example of such a preventive therapeutic approach is the testing of "preventive agents" (i.e., agents that prevent a malignant lesion from developing in a high risk population or which reverses pre-invasive neoplastic lesions which have developed in an at risk population). For example, a preventive agent may cause the resolution or prevention of dysplastic leukoplakia in abusers of tobacco [15,51], hence the prevention of the development of squamous cell carcinoma of the oral cavity.

1.5. Determination of clinical outcome or prognosis

Besides the diagnosis or subtype of a neoplastic process, many efforts have been made to determine the clinical aggressiveness of malignant lesions. These efforts to categorize the aggressiveness of cancers began with determining the stage and grade of cancers. This was the original basis for the staging of cancers; however, with the utilization of molecular analysis, currently stage is not always the controlling variable in determining clinical outcome [52]. In the case of prostate cancer, Gleason developed a score based on histopathologic features that ranged from 1 to 5 for a single focus of adenocarcinoma or 2 to 10 based upon both the primary and secondary patterns of cancer growth. Low scoring cancers (Gleason combined score $\leqslant 6$) usually have indolent courses, high scoring cancers (score $\geqslant 8$) usually have aggressive outcomes while intermediate scoring cancers, Gleason 7, cannot be easily predicted as to their aggressiveness. Similarly in breast cancer, the modified Bloom and Richardson grading method

as well as cytomorphometric analysis have both proved useful in identifying aggressive subtypes of ductal carcinomas [53]. For many cancers, the grade of the cells of the cancer is quite important in outcome.

As discussed, biomarkers have recently been identified that are used in subtyping carcinomas based upon the extent of expression of multiple biomarkers. Specifically, the ER^+ Type 1 subtype of breast cancer has been identified as having an excellent outcome; in contrast, breast cancers that do not express estrogen receptor, progesterone receptor and $p185^{erbB-2}$ have a poor outcome even though a cancer with high $p185^{erbB-2}$, if untreated, has a poor outcome. For treated lesions, the lack of expression of $p185^{erbB-2}$ takes away the option of therapy with Herceptin [43–47]. Also, our results in colorectal cancer in proximal carcinomas in Caucasian-Americans indicate those cancers expressing Bcl-2 (high), $p27^{Kip-1}$ (high) and no nuclear accumulation of p53 have relatively good outcomes [16,20,28].

Epigenetic changes also may affect the aggressiveness of neoplastic processes. As we begin to understand more about epigenetic changes and the development of cancer, increased molecular changes associated with epigenetics have been associated with subsets of cancers that are more or less aggressive. Some epigenetic changes such as methylation of CpG islands associated with the aggressiveness of cancers depend on increased expression of DNA methyl transferase in prostate cancer and increased histone methyl transferase such as EZH2 in breast and prostate cancers [54].

The characteristics of most malignant processes may vary with race and ethnic background. For example, the incidence and aggressiveness of prostate cancer is increased in African-Americans (AA) compared to Caucasian Americans (CA), but the incidence in Hispanic Americans (HA) is less than CA. The usefulness of using specific biomarkers for predicting the aggressiveness of tumors also may vary with race. For example, in colon adenocarcinoma, the nuclear accumulation of p53 as determined by immunohistochemistry is a strong prognostic biomarker for proximal colonic carcinoma, but p53 is not a useful prognostic biomarker in AAs [28]; for African-Americans, malignant cells in colorectal cancer with high grade are a prognostic indicator of very poor outcome [5]. Similarly, biomarkers of clinical outcome of breast cancer have been reported to vary with race [9,10,55].

1.6. Risk assessment

Patients whose genotypes (germ line DNA) carry specific mutations are at risk for developing specific types of cancers. If a risk of developing a cancer is high, then screening methods using biomarkers may be useful even when these same screening methods are a waste of resources for patients with lower risk. For example, children and adults who are from families with multiple endocrine neoplasia Type 2 (MEN2) and carry a mutation in the RET oncogene may be monitored for the occurrence of medullary carcinoma of the thyroid by periodically measuring calcitonin. In contrast, adults carrying a germ line mutation in enzymes that repair DNA mismatches (e.g., MLH2 and MSH6) have an increased risk for several neoplastic processes, especially adenocarcinomas of the proximal colon as do patients that carry a mutated copy of the APC gene. Besides germ line mutations which alone carry an increased risk for the development of specific neoplastic processes, certain other inherited genes result in an increased risk of developing specific cancers when combined with environmental exposures. For example, in a heavy smoker who has a glutathione S-transferase polymorphism in which GSTM1 is reduced has an increased risk for lung cancer [56]. Similar variations in acetylation ability secondary to variants of N-acetyltransferase (NAT) not only affect drug metabolism and hence the effectiveness and toxicity of specific drugs, but also increase the risk for some cancers, especially urothelial cancers of the bladder [57].

Similarly, epigenetic changes in the methylation of specific genes at CpG islands have major effects in the development of early neoplastic changes. Frequently, the methylated CpG islands involve important regulatory elements of suppressor genes such as p16 and of those genes involved in mismatch repair such as MLH1, in apoptosis, cell adhesion (E-cadherin) and in cellular migration (TIMPs); such patterns of methylation result in increased risk for specific cancers.

1.7. Targets for novel therapies

As our understanding of various pathways of cellular signaling and function have expanded and our knowledge of how these pathways are involved in the development and progression of neoplasia, important steps in these pathways are now targeted as primary or secondary forms of therapy for specific neoplastic processes. One of the initial targets was $p185^{erbB-2}$ or HER2/neu. A monoclonal antibody was developed against $p185^{erbB-2}$ (Trastuzumab or Herceptin) and it was found to inhibit effectively the functioning of $p185^{erbB-2}$ and to be effective against breast cancers (and subsequently other cancers) that over-express

Table 2
Monoclonal antibodies used to target signal pathways in cancer

Molecular target	Name	Commercial name	Cancers targeted	Comments
EGFr	Cetuximab	Erbitux	Oral, non-small cell lung	Non-small cell carcinoma of the lung with EGFr activation. Oral cancer together with radiation
Her-2/neu	Trastuzumab	Herceptin	Breast	
VEGF	Bevacizumab	Avastin	Colorectal; breast	
CD20	Rituximab	Rituxan	Lymphoid	
CD20	Ibritumomab tiuxetan* Rituximab	Zevalen	B cell lymphoma	
CD26	Tositumomab**	Bexxar	B cell lymphoma	
CD33	Gemtuzumab ozogamicin (monoclonal antibody combined with caicheamicin, an antitumor antibiotic)	Mylotary	Acute myelogenous leukemia	
CD52	Alemtuzumab	Campath	Chronic lymphoid leukemia	

*Frequently labeled with Y-90; **Labeled with I-131.

genes coding for p185^{erbB-2}. Therapy with Herceptin is also now approved for treating breast cancers which overexpresses p185^{erbB-2}.

The EGFr pathway can similarly be targeted by antibodies to EGFr (e.g., Cetuximab). Because almost all SCCs of the oral cavity express large amounts of EGFr on their cell surface membranes, Cetuximab has proved very effective in combined therapy with radiation in squamous lesions of the oral cavity [58]. Antibodies to EGFr have also been evaluated as therapeutic agents for cancers of the lung. They have proved primarily effective in only a small proportion of lung adenocarcinomas (especially bronchoalveolar type, now designated a form of intraepithelial neoplasia leading to adenocarcinoma of the lung) that have activating mutations or overexpression of EGFr [59,60]. In addition, multiple monoclonal antibodies have been developed to target specific molecules or processes e.g., inflammation, thought to provide a stimulating input to various cancers or to the development of the vasculature of cancers (Table 2) and these and many additional such antibodies are now in various stages of testing for a wide range of tumors [7,8,12,14].

Other molecules, many of which are designated small (inhibitory) molecules (e.g., $<$ 50D in molecular weight) have been developed to target the functional aspects of endogenous molecules or pathways thought to stimulate the growth of cancers. For example, gefitinib (Iressa) was developed to target the phosphokinase functions of EGFr; however, when tested as to its effectiveness against non-small cell lung cancers (NSCLC), like cetuximab, it required a tyrosine-kinase mutation in EGFr and/or some dysregulation or overexpression of EGFr expression for the therapy to be successful. However, a similar phosphokinase inhibitor of EGFr, erlotinib (Tarceva) has been approved for treatment of NSCLC.

Another small molecule developed to target a transduction system is Gleevec which targets the tyrosine-kinase domain of the fusion protein bcr:abl and the ATP binding pocket of KIT and PDGFRA. CML which frequently has the fusion protein bcr:abl is usually responsive to imatinib or the related kinase inhibitor dasatinib. Also, neoplastic lesions, especially gastrointestinal stromal tumors (GIST), with mutations in KIT or PDGFRA are usually at least transiently responsive to imatinib.

As described above, targeting of growth factors or their receptors with monoclonal antibodies or using small molecules to inhibit signal transduction pathways of growth factors aids in therapy of some neoplastic processes. Table 3 lists small inhibitory molecules that target signal transduction pathways.

Thus, translational research to identify therapeutic approaches which target signal transduction pathways which modulate specific neoplastic processes has been successful for multiple types of tumors. This area of translational research is rapidly expanding and is extremely promising.

1.8. Patterns of molecular expression to predict therapeutic responsiveness (prediction)

An important goal of translational pathology is to be able to use molecular features of a type of neoplasia to predict its responsiveness to specific therapies. Obviously, if the therapy targets a known biological feature, the presence of that biological feature is usually needed

Table 3
Small molecules/specific molecules used to target aspects of signal pathways in cancer

Signal transduction pathway target	Therapeutic small molecule	Commercial name	Molecular target	Cancer targeted	Comments
EGFr	Gefitinib	Iressa	Tyrosine kinase – EGFr	Bronchoalveolar carcinoma in Japanese women	Only activated EGFr; not on market
EGFr	Erlotinib	Tarceva	Tyrosine kinase – EGFr	Non-small cell lung/ pancreas	In combination with gemcitabine for pancreas
Kit, PDGFR, Bcr:abl	Imatinib	Gleevec	Tyrosine kinase of c kit and PDGFR, fusion protein, bcr:abl	GIST, CML	Response is frequently transient
Bcr:abl, SRC	Dasatinib	Sprycel	Tyrosine kinase	CML	Works on some patients resistant to imatinib
VEGFR – 2 & 3, FLT-3, Kit, PDGFR-β, Fms, craf, braf	Sorafenib	Nexavar	Tyrosine kinase of target pathway	Renal	
PDGFR-α & β, VEGFR1, 2, 3, Kit, FLT-3, CSF-1, RET	sunitinib	Sutent	Multiple kinases of pathway	GIST, renal	
GnRH	Abarelix	Plenaxis	Antagonist to GnRH receptor	Breast and prostate	
CD25	denileukin; diftitox	Ontak	CD25 component of il-2 receptor	Cutaneous T cell lymphoma	
CXCR1	Repertaxin	Repertaxin	il-8 receptor	All	Reduces inflammation mediated by il-8

for successful therapy. For example, proliferation may be turned on and targeted to be turned off or apoptosis may be turned off and targeted to turn on. Similarly, if a specific molecule or pathway is targeted by a therapy, the presence of that molecule at a specific level may be necessary for the therapy to be successful. Sometimes the molecule targeted must be "turned-on" or constitutively active for the therapy to be successful, such as EGFr in the therapy for NSCLC; however, rarely some therapy such as therapy with the TRA-8 antibody to the death receptor, DR5, is not based in this case on target levels of DR5 [12,14]. Thus, some types of therapy may work independent of high levels of the molecules which are thought to be targets of the therapy. In such cases, it would be useful to be able to identify molecular features which can be correlated with the potential effectiveness of a therapy. This area of translational research is very early in its development, but it is potentially very important.

1.9. Imaging

Techniques which image neoplastic lesions have improved greatly over the last three decades, especially in the modalities of imaging and the sensitivity of imaging, including the minimum size of lesions that can be imaged. Imaging, including x-ray computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), positron emission tomography (PET), single-photon emission computerized tomography (SPET), and bioluminescence and fluorescence optical imaging, can be used successfully in identifying primary or metastatic lesions (i.e., staging of disease) and to separate benign from malignant lesions. These imaging models, e.g., ultrasound, can be used to detect neoplastic nodules and hence guide fine needles for aspiration and thus to aid in early detection/diagnosis [11]. Other uses of imaging methods include determining the effectiveness of specific therapies, evaluating the pharmokinetics of therapeutic drugs, predicting drug resistance, determining tumor vascularity of neoplastic lesions, evaluating novel therapies in animal models and aids in evaluating gene therapy [7,8,61,62]. Imaging can be used to evaluate neoplastic processes including the evaluation of the extent of tumor metabolism using labeled glucose transport analogues. Also agents for imaging that correlate with proliferation and apoptosis are under development. In general, imaging, although a relatively new area of translational research, is a major growth area, as many of the clinical targets for therapy can also be targeted for imaging.

2. Challenges and advances in translational pathology

Several areas of translational pathology use immunohistochemistry in evaluations. For example, the determination of estrogen receptor and progesterone receptors in the diagnosis/prognosis of breast cancers as well as erbB-2 expression to verify the target for Herceptin therapy can be measured by this technique. The collection and processing of tissues can have a great effect on immunohistochemistry [63]; yet the effects on immunohistochemistry of collection and processing of tissues are inadequately understood. Specifically both the fixation and the processing of tissues to paraffin blocks may greatly affect immunohistochemistry [64, 65]. In addition, standardized methods of performing immunohistochemistry have not been accepted [66]. For example, some antigens (e.g., pAkt) rapidly decay when exposed to ischemia while the phenotypic expression of other proteins (e.g., heat shock proteins) may be increased by warm ischemia (reviewed in [67]).

Over the years there has been a great concern that the degradation of mRNA will be limiting in the use of human tissues to identify molecular features of neoplastic lesions. Our current knowledge indicates that the more unstable types of mRNA are most likely degraded during the period of warm ischemia, i.e., between compromise of the vascular supply to an area of tissue and the removal of the tissue. One of the advances in evaluating translational pathology is the use of amplicons of < 100 bp in the analysis of gene expression using real time quantitative PCR [68,69]. Specifically, our results and those of others indicate that the use of small amplicons permits the successful utilization of partially degraded mRNA in studies of gene expression in tissues that have extended periods of warm ischemia. As the speed of complete sequencing of genes increases as its costs decrease, full sequencing is likely to replace PCR in analysis. Of concern will be the quality of the mRNA and the effects of partial degradation of mRNA on the results of sequencing. These issues are now being evaluated.

In summary, evaluating the translational pathology of neoplastic processes has been greatly slowed by a lack of understanding of the effects of handling of human tissues on molecular methods as well as attention to the quality of human tissues used in assays. In addition, the methodology used in analysis has not been standardized. This creates problems in the literature, especially for molecules for which there are multiple splice variants and different patterns of staining depending if antibodies are to the C or N tail of the antigen.

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GENE METHYLATION BIOMARKER ANALYSIS OF PROSTATE BIOPSIES FROM MEN WITH 1 OF 12 CORES POSITIVE FOR CANCER: GREATER METHYLATION PREVALENCE AND EXTENT IN GLEASON 7 THAN GLEASON 6 CANCER

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INTRODUCTION AND OBJECTIVES: Many studies have shown that tumor-associated changes in DNA methylation can act as biomarkers for the presence of prostate cancer. We and others have also shown that changes in DNA methylation patterns often occur in histologically benign tissues adjacent to cancer, thus acting as “field effect" biomarkers. Field effect biomarkers have been used in tests to detect prostate cancer missed due to biopsy (bx) sampling error. Bx sampling error can also result in underestimates of prostate cancer stage or grade. Such underestimates are of concern as more men are enrolling in Watchful Waiting Active Surveillance programs based on a diagnosis of limited, “low risk” prostate cancer involving 1 or 2 bx cores and Gleason score 6 or less. In this study, we used three established DNA methylation markers of prostate cancer (GSTPi, APC and RAR-beta) to evaluate all of the diagnostic bx cores from a series of patients with 1 of 12 cores positive for cancer. Our objective was to look for field effects that may provide a useful index of higher grade malignancy in the adjacent tissues
METHODS: DNA was extracted from prostate bx tissue prints and used for quantitative methylation-specific PCR (QMSP) assays of GSTPi, APC and/or RAR-beta promoter region methylation. All testing was done blinded. Each of 12 diagnostic bx cores were tested from a series of twenty-two patients: 12 patients with Gleason score (GS) 3+3 in 1 of 12 cores and 10 patients with minimal GS 7 in 1 of 12 cores.

RESULTS:
In 7 of the 10 cases (70%) where the 1 cancer core showed GS 7, all 3 DNA methylation markers were positive for field effects that extended into one or more adjacent histologically benign bx's. In contrast, only 3 of 12 cases (25%) with 1 core GS 6 cancer were field effect positive for all 3 methylation markers; interestingly 2 of these 3 cases were subsequently diagnosed with GS 3+4 cancer at radical prostatectomy (RP). GSTPi showed the best performance as a single marker “field effect” test for adjacent high grade cancer; GSTPi was positive in 4 of the 12 1-core GS 6 cases (including the 2 cases found to have GS 7 cancer at RP), and in 8 of the 10 1-core GS 7 cases.

CONCLUSIONS:
GSTPi, APC and/or RAR-beta DNA promoter region methylation was observed to be more prevalent and more extensive in histologically benign bx tissue from men with one core positive for GS 7 cancer, as compared with one core GS 6. These “field effect” biomarkers might be useful for predicting the presenceof occult cancer with aggressive characteristics not detected using standard biopsy histopathology.

that distinguish CC from UC. These features are independent of the tissue of origin and represent disease specific markers. Some of these signatures were only found in the colonic mucosa (m/z 8413, 3666 & 3595) or submucosa (m/z 4122, 8774, 2778, 9232 & 9519) while others were found in both two layers (m/z 5045, 6139 & 9245). This information may provide new avenues for the development of novel diagnostic, prognostic and therapeutic targets. We will analyze CRAC in IBD segments2,3 to look for these proteins that may help in studying their biological mechanisms in cancer transformation.

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Biomarkers: Biomarkers of Risk and Surrogate Endpoints

A46 Genomic ancestry is associated with risk group and survival in children with neuroblastoma. Navin R. Pinto[1], Eric R. Gamazon[1], Anuar Konkashbaev[1], Nancy J. Cox[1], M. Eileen Dolan[1], Sharon J. Diskin[2], Wendy B. London[3], Marcella Devoto[2], John M. Maris[2], Susan L. Cohn[1]. [1]University of Chicago, Chicago, IL, [2]University of Pennsylvania, Philadelphia, PA, [3]Harvard University, Boston, MA.

Background: In a cohort of 3539 children with neuroblastoma, we have previously reported that self-reported black race is significantly associated with high-risk disease ($p < 0.001$) and late relapse or death ($p = 0.04$) [1]. To investigate if the component of genomic variation that co-segregates with African ancestry is associated with risk group and survival in neuroblastoma, we interrogated genome-wide SNP genotypes against those phenotypes in 3180 neuroblastoma patients.

Methods: Genome-wide SNP genotypes from 3404 children with neuroblastoma were obtained from 3 Illumina platforms: HumanHap550 v1, HumanHap550 v3 and HumanQuad 610. Quality filtering removed 17 patients who were genotyped twice, 15 patients with ambiguous sex, 72 patients with some degree of relatedness and 120 patients with poor genotype call rates (defined as >5% total SNPs interrogated without a genotype call). Genotypes from the remaining 3180 patients were included in the analysis. Using ancestral reference populations from the International HapMap Project (Caucasian - CEU, African - YRI and Asian – CHB/JPT) and the EIGENSTRAT method, a principal components analysis for each of the three genotyping platforms was performed and an ancestry map of all patients was created. Pricincipal Component 2 (PC2), which separated black neuroblastoma patients and the YRI HapMap samples from all other ethnic groups, was used as a continuous variable for linear regressions with both event-free survival and risk group.

Results: PC2 was correlated with event-free survival in patients with intermediate- and high-risk neuroblastoma (those patients that receive chemotherapy), showing that degree of African ancestry was associated poorer outcome ($p = 0.047$). Furthermore, linear regression of PC2 versus risk group classification showed that degree of African ancestry was associated with high-risk disease ($p = 0.05$).

Discussion: Here we show that the component of genomic variation that co-segregates with African ancestry is associated with high-risk disease and a poorer event-free survival. We hypothesize that germline genetic variants associated with both high-risk neuroblastoma and event-free survival after chemotherapy will have different allelic frequencies in Caucasian and African populations and that patients of African ancestry may also harbor unique genetic variants associated with high-risk disease and event-free survival not found in Caucasian patients. Efforts to identify these genetic variants are underway. These results underscore the need to perform association studies from a variety of ancestries, as each group may yield novel associations.

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Biomarkers: Novel Technologies

A47 Prostate biopsy tissue print technologies: A practical and innovative approach to overcoming racial disparities in the datasets used for prostate cancer biomarker development. Sandra M. Gaston[1], Gary P. Kearney[1], William E. Grizzle[2]. [1]New England Baptist Hospital, Boston, MA, [2]University of Alabama, Birmingham, AL.

Health disparities can be unintentionally aggravated by patterns of medical practice that shape biomedical research. In prostate cancer research, radical prostatectomy specimens have been the major source of frozen tissue samples used for the molecular genetic analysis of primary prostate cancers. However, because men who are diagnosed with relatively advanced prostate cancer are more likely to be offered hormonal and/or radiation treatment, the patients who are diagnosed too late to be offered surgery are underrepresented in conventional tissue banks that depend upon radical prostatectomy specimens. As a result, the prostate cancer patients who are diagnosed with the most serious forms of this disease are significantly underrepresented in the gene expression datasets that have become central to biomarker and therapeutic target discovery. African American men are particularly impacted by this under-representation, both because they are more likely to be diagnosed with relatively advanced prostate cancer and because of a preference in many African American communities for non-surgical forms of prostate cancer treatment.

Potentially, a more representative collection of samples could be obtained from newly diagnosed, untreated prostate cancers by utilizing the diagnostic prostate biopsy specimens. The prostate biopsy population includes the entire range of newly diagnosed cancers; it also includes all of the patients whose biopsies are cancer-negative. However, there are well recognized barriers to obtaining samples from diagnostic prostate biopsies for research. In most settings, each prostate biopsy core must be processed in its entirety as a formalin-fixed paraffin embedded (FFPE) specimen and the remnant FFPE biopsy tissue is often relatively limited and potentially compromised by fixation and processing. We have overcome

the barriers to using routine diagnostic biopsies for molecular biomarker studies by developing a set of tissue printing technologies that allow us to obtain high quality DNA and RNA from each biopsy core without compromising pathology diagnosis. These tissue printing technologies have now been successfully applied to a range of cancer studies, including molecular analyses of metastatic lesions which are frequently available only as needle biopsies.

We have formed a collaboration in which tissue print technologies are used to support a systematic comparison of molecular biomarker patterns in prostate biopsies obtained from African American and European American patients. Our data analysis plan includes an evaluation of marker associations with both self-identified ancestry and with genetic lineage as defined by ancestry informative markers. This approach has been particularly interesting in the evaluation of DNA methylation markers, where it has been demonstrated that the extent of cancer-associated hypermethylation depends in part on the DNA sequence of the marker allele. More generally, because the use of biopsy samples allows us to obtain a more representative sample of the African Americans and European American patient populations, we can improve the pre-clinical assessment of potential strengths and weaknesses of specific biomarker tests as tools for guiding patient care

Cancer Treatment and Outcomes: Drug Design, Discovery, and Delivery

A48 Cost-effective topical delivery of chemotherapy to treat cervical dysplasia and prevent progression to HPV-associated cervical cancer in the low-resource setting.
Stephen I-Hong Hsu, Michael Wood, Edward J. Wilkinson. University of Florida College of Medicine, Gainesville, FL.

Introduction: Cervical cancer is the second most commonly diagnosed cancer and the third leading cause of death by cancer in women worldwide. Over 85% of annual cervical cancer deaths occur in developing parts of the world and other low-resource populations where it is the leading cause of death from cancer among women chronically infected by at least one oncogenic type of human papillomavirus.

Background/Significance: Pap smear cytologic screening and current therapies for pre-cancerous low-grade lesions are costly, invasive and not feasible for use as therapy in low-resource settings. Inexpensive and validated alternative screening methods such as visual inspection with acetic acid (VIA) and a promising new HPV-DNA test (*care*HPV™, Qiagen), suggest the feasibility of implementing a single visit "screen-treat-prevent" public health care model in low-resource settings. However, there remains an unmet and urgent global need for an alternative therapy to serve as the "treatment arm" of a combined strategy that integrates screening with a safe, effective and affordable treatment for cervical dysplasia to prevent progression to cervical cancer.

Purpose/Approach: Our aim is to create a non-invasive therapy specifically for use in a "screen-treat-prevent" public health care model that can be readily implemented even in a remote village in Sub-Saharan Africa, without benefit of electricity, running water, clinical work-space or formally trained medical personnel. Such a therapy should be inexpensive, easily self-administered and curative as a single dose therapy (assume no follow-up). We used an approach derived from a fundamental principle of synthetic biology—a "bottoms up" approach—employing highly sophisticated material science research instrumentation and technologies to engineer a "low tech" alternative therapy that represents an unconventional and innovative solution to an urgent global health challenge.

Results: Our preliminary studies demonstrate the feasibility of controlled delivery of decoy peptide *in situ* to the cervical transformation zone in a transgenic mouse strain (K14E6) in which the high-risk type HPV16 E6 oncoprotein synergizes with 17 -estradiol to induce disease *in situ* that closely mimics the multi-stage development of human cervical cancer. Stable peptide-containing biopolymer formulations were created with properties that allow them to be solid phase at room temperature for ease of targeted application to the ectocervix, instantly melt at body temperature, adhere to mucosa and enhance peptide penetration across cervical mucosa. Penetration of decoy peptide across multiple layers of cervical squamous epithelium occurred in a dose- and time-dependent manner.

Social Impact: The ability to offer women a health care solution that encompasses a "screen-treat-prevent" approach requiring minimal medical supervision is at once powerful and empowering. Advancement in modern western medicine should aim at producing more equity rather than disparity. Studies have shown that women are often caregivers of the communities to which they belong, so that when they achieve improvements in their own health, the quality of life of the community as a whole improves significantly. This study hopes to add to the effort of continuing to enable and empower women all over the world to take stewardship of their own health for the betterment of the community at large.

Controlled topical delivery of biopolymer drug formulations represents an affordable and effective therapy for cervical dysplasia aimed at reducing cervical cancer incidence and mortality. Optimization of drug delivery using biopolymer formulations in a mouse model of human multi-stage cervical cancer will lay the groundwork for future human clinical trials.

A49 Multifunctional nanocarriers against cervical cancer.
Mary R. Saunders, David J. Olivos, Ryan G. Lim, Lorenzo Berti, Kit S. Lam. University of California, Davis, CA.

Cervical cancer accounts for 500,000 new cases and 250,000 deaths in women worldwide. In low-income countries, cervical cancer is the most common cause of cancer-related deaths among women. Novel approaches for the early detection and aggressive treatment of cervical cancer are urgently needed to increase the survival rates. To this end, a promising strategy that might improve the specificity of current diagnostic and therapeutic agents is the development of drugs or contrast agents targeting glycans, polysaccarides decorating normal and cancerous cells that provide a unique fingerprint on the cell surface. Tumor glycans are important in regulating pivotal events during the development and progression of cancer. Changes in their sequence and structure are known to be responsible for cell proliferation, invasion, angiogenesis, and metastasis. Malignant transformation of cervical cells is accompanied by the expression of specific glycans that are absent on normal uterine cervix cells. Peptide-based ligands specific for these cervical cancer glycans have been discovered, and their use as tumor-homing agents for diagnostics and therapeutic purposes has been explored in xenograph models. We have currently developed an innovative drug delivery vehicle for the simultaneous and highly specific imaging and treatment of cervical cancer. To take full advantage of the enhanced permeability and retention effect (EPR) of the nanocarriers and to have deep penetration inside the tumor mass, we have developed a telodendrimer system, comprised of clusters of oligomeric cholic acids (CA) linked to a polyethylene glycol (PEG) chain which self-assemble within an aqueous